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(54) Title: PRRSV ANTIGENIC SITES IDENTIFYING PEPTIDE SEQUENCES OF PRRS VIRUS FOR USE IN VACCINES OR DIAGNOSTIC ASSAYS

(57) Abstract

The invention provides antigenic sites of PRRSV isolates. The antigenic sites are neutralizing, conserved, non–conserved and conformational, can elicit antibodies and are found on protein GP_4 and N encoded by ORF4 and ORF7 of PRRSV. The peptide sequences identified by the sites can be incorporated in vaccines directed against PRRS and in diagnostic tests for PRRS. Also, discriminating tests can be developed that can be used next to marker vaccines in programs designed to eradicate PRRS from pig herds.

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Title:PRRSV antigenic sites identifying peptide sequences of PRRS virus for use in vaccines or diagnostic assays

The invention relates to the causative agent of Mystery Swine Disease, the PRRS virus, to peptide sequences identified in the PRRS virus, and to incorporating these sequences in vaccines and diagnostic tests.

PRRS virus (PRRSV) is the causative agent of a pig disease, currently called porcine reproductive and respiratory syndrome (PRRS). The virus is the causative agent of a pig disease, seen since approximately 1987 in the US and since 1990 in Europe, known initially under various names such as Mystery Swine Disease, Swine Infertility and Respiratory Syndrome, and many more. The virus itself was also given many names, among which Lelystad virus (LV), SIRS virus, and many more, but is now mostly designated porcine reproductive and respiratory syndrome virus (PRRSV). It causes abortions and respiratory distress in pigs and was first isolated in Europe in 1991 (EP patent 587780, US patent 5,620,691) and subsequently in the US and many other countries throughout the world. PRRSV is a small enveloped virus containing a positive strand RNA genome. PRRSV preferentially grows in macrophages. In addition to macrophages, PRRSV can grow in cell line CL2621 and other cell lines cloned from the monkey kidney cell line MA-104 (Benfield *et al.*, J. Vet. Diagn. Invest. 4; 127-133, 1992). The genome of PRRSV, a polyadenylated RNA of approximately 15 kb was sequenced in 1993 (Meulenberg *et al.*, Virology 192; 62-74, 1993). The nucleotide sequence, genome organization and replication strategy indicated that PRRSV is related to a group of small enveloped positive-strand RNA viruses, designated Arteriviruses. This group includes lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). These viruses have a similar genome organization, replication

strategy, morphology, and amino acid sequence of viral proteins. Arteriviruses contain a genome of 12.5 to 15 kb and synthesize a 3' nested set of six subgenomic RNAs during replication. These subgenomic RNAs contain a leader sequence which is derived from the 5' end of the viral genome. ORFs 1a and 1b comprise approximately two thirds of the viral genome and encode the RNA dependent RNA polymerase. Six smaller ORFs, ORFs 2 to 7, are located at the 3' end of the viral genome. ORFs 2 to 6 likely encode envelope proteins whereas ORF7 encodes the nucleocapsid protein (Meulenberg *et al*, Virology 206; 155-163, 1995).

PRRSV is the first Arterivirus for which it has been demonstrated that all six proteins encoded by ORFs 2 to 7 are associated with the virion. The 15-kDa N protein (encoded by ORF7) and the 18-kDa integral membrane protein M (ORF6) are not N-glycosylated, whereas the 29- to 30-kDa GP_2 protein (ORF2), the 45- to 50-kDa protein GP_3 protein (ORF3), the 31- to 35-kDa GP_4 protein (ORF4), and the 25-kDa protein GP_5 (ORF5) are. These proteins have also been detected in extracellular virus and lysates of cells infected with a North American isolate of PRRSV, ATCC-VR2332, and other isolates of PRRSV (other isolates of PRRSV are for example CNCM I-1140, ECACC V93070108, CNCM I-1387, CNCM I-1388, ATCC-VR2402, ATCC-VR2429. ATCC-VR2430, ATCC-VR2431, ATCC-VR2475, ATCC-VR2385, but many others are known).

We earlier described the isolation and characterization of a panel of PRRSV-specific MAbs that were specific for GP_3, GP_4, M and N (van Nieuwstadt *et al.*, J. Virol. 70, 4767-4772, 1996). Interestingly, MAbs directed against GP_4 were neutralizing, suggesting that at least part of the protein is exposed at the virion surface. Furthermore, most of the Mabs directed against N reacted with all PRRSV isolates tested.

PRRS in it self is a problem of major concern for the swine industry in most parts of the world. Introduction of PRRSV in

pig herds will cause severe economic losses. Diagnostic testing against PRRS is widely practiced by many veterinarians and laboratories. Most diagnostic tests, such as IPMA, IFT, IFA, ELISA, each comprising suitable means of detection such as conjugated enzymes or fluorochromes, and other substrates, use interactions between antigen derived from PRRSV and antibodies directed against PRRSV to measure the presence of either PRRSV antigen or antibodies directed against PRRSV in a biological sample, such as blood, serum, tissue, tissue fluids, lavage fluids, urine, faeces, that is sampled from the animal (such as a pig) to be tested. The antigen and/or antibodies used in these diagnostic tests, or diagnostic kits or assays, for PRRS diagnosis are only defined by their origin from, or by their reactivity with PRRSV. In principle this suffices for screening assays where a high specificity or sensitivity is not explicitly required. However, the ever continuing spread of PRRS has caused great concern among the pig industry, to the extent that it is deemed needed to eradicate PRRS from whole herds, or even from complete areas, regions, or countries where pigs are raised. A clear example of this need is the proposed eradication program relating to PRRS in Denmark. If one decides to completely eradicate PRRS then diagnostic tests are needed that exhibit higher specificity or sensitivity than the tests used today.

Vaccination against PRRS is also widely practiced. Several examples are known of modified live vaccines that are used, and also killed vaccines are known. However, a problem with live vaccines in general, and thus also with live PRRS vaccines, exists in that these vaccines have a tendency to spread to non-vaccinated pigs, thereby spreading instead of reducing detectable infection in pig herds, and thus being counter productive to complete eradication. If a line marker vaccine were used that could serologically be differentiated from the wild type virus, then this problem would be greatly reduced. Added disadvantages are that live vaccines sometimes cause anaphylactic reactions in the vaccinated pigs, because

of undefined antigenic components. Although killed vaccines in general are reported to induce protection in the vaccinated pig, and have the additional advantage that they do not spread from pig to pig, a disadvantage of killed vaccines is that it may be hard to accrue sufficient antigenic mass in one dose of a vaccine to elicit a measurable and protective immune response. Especially killed vaccines that can induce measurable neutralizing antibody titers in pigs would be beneficial to have since measuring these neutralizing antibodies in vaccinated pig populations would help generate understanding about the level of protection obtained by vaccination in the pig herd. In addition, if one succeeds in assembling the necessary antigenic mass, this also means that more and other undefined antigenic mass is also present in the vaccine, which can also give rise to the anaphylactic reactions as described above. In this sense it would be beneficial to know which specific site on PRRSV is involved in neutralization, leading to the design of better suited vaccines, incorporating the important peptide sequences needed for eliciting neutralizing antibodies. An advantage of the currently used vaccines originating from PRRSV isolates isolated in the US is that such vaccines, albeit fully protective against and immunologically cross-reactive with European isolates of PRRSV, contain, as yet undefined, epitopes or antigenic sites by which they can be discerned from European isolates of PRRSV. Reciprocally, live vaccines originating from PRRSV isolates isolated in Europe, albeit fully protective against and immunologically cross-reactive with US isolates of PRRSV, contain similar as yet undefined epitopes or antigenic sites by which they can be discerned from US isolates of PRRSV.

If serological tests would be available which could discriminate (based on the small epitopic differences between PRRSV isolates) between pigs that are either vaccinated with a US derived vaccine or infected with a European wild type of PRRSV (being vaccinated or not), or which could discriminate pigs that are either vaccinated with a European derived

vaccine or infected with an US wild type of PRRSV (being vaccinated or no), than marker vaccines and corresponding diagnostic tests (incorporating said discerning epitopes or antigenic sites) could be developed which could be used with large confidence in eradication programs for PRRS. For example, in Denmark it would than be possible to vaccinate with a US derived vaccine and measure the set of antibodies in the Danish pigs which are solely directed against unique epitopes on European wild types of PRRSV and not cross-reactive with US strains. This would enable the unequivocal detection and subsequent removal of wild type infected pigs from Danish herds. Currently, such a discrimination is not possible due to the overall broad immunological cross-reactivity between PRRSV isolates. It goes without saying that such combined vaccination-testing programs will be the basis for eradication of PRRS, and can also be used in other countries, if needed with distinct PRRSV antigenic sites being used in vaccine and/or diagnostic test.

The invention now provides antigenic sites comprising peptide sequences of PRRSV which allow the improvement of vaccines, be it killed or attenuated vaccines or vaccines derived via recombinant DNA technology, and antigenic sites which allow the improvement of diagnostic methods, tests and kits and the production of new diagnostic methods, tests and kits. Artificial changes or amino acid residue substitutions that maintain the antigenicity (as for example defined by the reactivity with polyclonal sera or MAbs) and thus functionality of the antigenic site can easily be derived from sequences known to constitute an antigenic site of a specific isolate by a person with ordinary skills in the art of peptide design and synthesis. For example, certain amino acid residues can conventionally be replaced by others of comparable nature, e.g. a basic residue by another basic residue, an acid by an acid, a bulky by a bulky, a hydrophobic or hydrophilic by another hydrophobic or hydrophilic residue, and so on. Also, other, less conventional but more specific changes are also possible that

maintain or even improve the antigenicity of the selected sequence. Such changes can for example be made by PEPSCAN based amino acid substitutions or replacement mapping techniques (van Amerongen et. al., Peptide Research (1992) 5, 269-274. In short, amino acid residues within the antigenic sites provided by the invention can e.g. be replaced conventionally or under guidance of replacement mapping, whereby the resulting peptide sequences are functionally equivalent to the antigenic site. The replacing amino acids can be either L- or D- amino acid residues. In addition, the peptide sequences provided by the invention are rendered even more immunogenic by conjugating them to adjuvants (such as KLH) known in the art. Additionally, the peptides are rendered even more immunogenic by making peptides with one (such as tandem peptides) or more repeated sequences or by polymerization or circularization.

Although it has been shown before that the N protein is immunogenic (Meulenberg (1995), J. Clin. Diagn. Lab. Immunol. 2, 652-656, GB 2 289 279 A) and that conserved and nonconserved regions between the N protein of European strains (LV) and US strains (VR2332) exist (WO 96/04010), we demonstrate here for the first time which conserved and nonconserved regions are antigenic and which can be used individually or in combinations as antigens for immunization or diagnostic assays. Furthermore it is identified here that the antigenic regions in the N protein consist both of linear and conformation dependent epitopes.

The GP4 protein is the first structural protein of PRRSV for which is shown that it elicits antibodies that can neutralize the virus. A specific region of approximately 40 amino acids was identified and defined that should be exposed at the virion surface as a target for neutralizing antibodies, which then prevent the virus to infect the cells. This is an exciting new finding since it is generally assumed that the GP5 protein, the major structural of PRRSV, is the most important candidate involved in the attachment of the host cell.

The invention provides a major antigenic site, a neutralization site on GP_4 of PRRSV. The invention provides the localization of a major neutralization site important for the design of effective marker vaccines that comprise amino acid core sequences and amino acid sequences flanking the core sequences of PRRSV isolates which sequences comprise the neutralization site on the ORF4 protein of PRRSV. By incorporating the relevant neutralization site sequences in the various types of vaccines, it is possible to specifically induce neutralizing antibodies in the vaccinated pig. Killed vaccines comprising the neutralization site provided by the invention are made to induce measurable neutralizing antibodies. Especially sequences located at positions in the ORF 4 encoded protein of PRRSV corresponding to those found at about amino acid 40 to 79 as found in PRRSV isolate I-1102 comprise the neutralization site. Furthermore, selected peptide sequences are made even more immunogenic by mixing the peptides with adjuvants or other carriers known in the art. The thus obtained peptide compositions are used as a vaccine. However, also the selected peptide sequences comprising the neutralization site are incorporated in vaccine vector systems, being either distinct recombinant vectors derived of heterologous viruses or bacteria, but the selected peptide sequences are also selectively incorporated in PRRSV vector viruses or vaccines derived thereof.

In a further aspect of the invention, amino acid sequences located at positions corresponding from about 52 to 75 more specifically constitute a broadly reactive neutralization site. Other embodiments of the neutralization site provided by the invention can be found among the various PRRSV isolates known or to be found (see for instance the experimental part of this description). It is easy for any person working in the field of molecular biology to compare the sequences comprising the neutralization site provided by the invention with the amino acid sequence of the ORF 4 encoded protein of yet another PRRSV isolate.

The invention also provides peptide sequences of PRRSV which improve diagnostic tests, be it antigen or antibody detection tests. The invention provides various groups of antigenic sites which are used alone or in combination in diagnostic tests. In this way diagnostic tests are provided by the invention that serve the various needs that exists in the field with regard to diagnosis and differential diagnosis. Antigen-antibody interactions always entail cross-reactive epitope-paratope interactions of amino acid sequences that are from 5 to 15 amino acid sequences long. Thus amino acid sequences of 5 to 15 amino acids long and partly or completely overlapping with the core sequences of the antigenic sites of invention are provided by the invention for incorporation in diagnostic tests. These peptide sequences are used to select or design antigen or antigenic substance containing the sequences in the test to be used. Alternatively, and provided by the invention, are synthetic antibodies reactive with the antigenic sites provided by the invention. These sites or related sequences react with synthetic antibody obtained from systems such as phage display libraries or clonal selection of (heavy chain) antibodies that constitute antibody-like molecules which can easily be expressed in heterologous expression systems.

One group provided by the invention comprises the peptide sequence corresponding to said neutralization site, as already explained above. Diagnostic tests comprising this site and/or antibodies specifically directed against this site detect neutralizing antibodies in the pig.
Another group provided by the invention comprises a conserved antigenic site on protein N. Within the conserved antigenic site the invention provides a core sequence VNQLCQLLGA or VNQLCQMLGK. Diagnostic tests comprising this site and/or antibodies specifically directed against this site detect those antibodies in pigs that specifically react with most PRRSV isolates. Also, diagnostic tests are provided that use antibodies directed against the conserved site to detect the

PRRSV antigen, thereby allowing the test to detect PRRSV isolates, irrespective of their origin.

Another group provided by the invention comprises a non-conserved differentiating antigenic site on protein N. Diagnostic tests comprising this site and/or antibodies specifically directed against this site detect those antibodies in pigs that specifically react with distinct PRRSV isolates, whereby for example vaccinated pigs can be discriminated from pigs infected with wild type PRRSV. Also, diagnostic tests are provided that use antibodies directed against the non-conserved site to detect the PRRSV antigen, thereby allowing the test to discern different PRRSV isolates. Within one such a non-conserved site the invention provides a core sequence PRGGQAKKKK or PRGGQAKRKK or PRGGQAKKRK or GPGKKNKKKN or GPGKKNKKKT or GPGKKNRKKN or GPGKKFKKKN or GPGKKIKKKN or GPGQINKKIN. Within another non-conserved site the invention provides a core sequence MAGKNQSQKK or MPNNNGKQTE or MPNNNGKQPK or MPNNNGKQQK or MPNNNGKQQN or MPNNNGKQQK or MPNNNGRQQK. Also, artificial changes that maintain the antigenicity and thus functionality of the above core sequences in the GP4 or N protein can easily be introduced by anyone skilled in the art of peptide design and synthesis, as described above.

The invention also provides a group comprising conformational epitopes (which vary greatly among the various isolates) which can be found at positions corresponding to those found in isolate I-1102 from amino acid position 51 to about 68 (in isolate I-1102 core sequence PKPHFPLAAEDDIRHHL) or from 79 to about 90 (in isolate I-1102 core sequence SIQTAFNQGAGT) or from 111 to 124 (in isolate I-1102 core sequence HTVRLIRVTSTSAS) on protein N. The conserved and non-conserved and differentiating and conformational sites in the N protein, which sites are provided by the invention, provide diagnostic tests that unequivocally diagnose PRRSV infections. Tests are made that avoid employing non-conserved sites thereby avoiding false-negative results. In addition, the various non-conserved sites are used in the development

of differentiating tests that can e.g. discriminate vaccinated pigs from pigs infected with wild type isolates of PRRSV. Again, as said it is easy for any person working in the field of molecular biology to align the sequences comprising the conserved or non-conserved or conformational epitope sites with amino acid sequences of the ORF 7 encoded protein of yet another PRRSV isolate. The sites provided by the invention are used in new pairs of vaccine-discriminating diagnostic tests for use in eradication programs of PRRS.

Experimental part

Materials and methods

Cells and viruses

The *Ter Huurne* strain (CNCM I-1102) of PRRSV was isolated in 1991 (Wensvoort *et al.*, 1991). The US ATCC-VR2332 strain was isolated by Benfield *et al.* (1992). Strain NL1 (Netherlands, 1991) was isolated in our lab, Strain NY2 (England, 1991) was kindly provided by T. Drew, strain DEN (Denmark, 1992) was kindly provided by A. Botner, strain LUX (Luxemburg, 1992) was kindly provided by Losch, SPA1 and SPA2 (Spain, 1992) were kindly provided by Shokouhi and Espuna, respectively, and strain FRA (France, 1992) was kindly provided by Y. Leforban.

PRRSV and VR2332 were grown on CL2621 cells as described previously (van Nieuwstadt *et al.*, 1996). The seven different European isolates were grown in porcine alveolar macrophages. Macrophages were maintained as described before (Wensvoort *et al*, 1991). BHK-21 cells were maintained in Dulbecco's Minimal Essential Medium supplemented with 5% fetal bovine serum and antibiotics. For transfection experiments, BHK-21 cells were grown in Glasgow Minimal Essential Medium (GIBCO-BRL/Life Technologies Ltd).

Antisera.

Porcine anti-PRRSV serum 21 and rabbit anti-peptide sera 698 and 700 were used in previous experiments. Serum 700 is directed against amino acids 106 to 122 (CLFYASEMSEKGFKVIF) encoded by ORF4 of PRRSV and was obtained from a rabbit. The production and characterization of MAbs have been described (van Nieuwstadt *et al*, 1996). The hybridomas were derived from five consecutive fusion experiments and directed against ORF 4 protein (MAb 121.4, 122.1, 122.12, 122.20, 122.29, 122.30, 122.59, 122.66, 122.68, 122.70, 122.71, 126.1, 126.7, 130.7, 138.28) or ORF 7 protein (MAb 122.17, 125.1, 126.9, 126.15, 130.2, 130.4, 131.7, 138.22, WBE1, WBE4, WBE5, WBE6, SDOW17) Mabs WBE were graciously provided by Dr. Drew, Weybridge, UK; Mab SDOW17 was graciously provided by Dr. Benfield, South Dakota, US.

Plasmid constructions

Two oligonucleotides located upstream (PRRSV13) and downstream (PRRSV14) of ORF4 have been used earlier to amplify and clone ORF4 of isolate I-1102 in pGEM-4Z using the BamHI and HindIII sites introduced in the primers (Meulenberg *et al*. 1995). The resulting plasmid was named pABV209. Two oligonucleotides located at a similar position with respect to the initiation codon (PRRSV4) and the termination codon (PRRSV5) of ORF4 of VR2332 were used to amplify ORF4 of VR2332 by means of RT-PCR as described in previous studies. The PCR fragment was digested with BamHI and partially with HindIII since ORF4 of VR2332 contains an internal HindIII site, and cloned in pGEM-4Z resulting in plasmid pABV270. Recombinant DNA techniques were performed essentially as described by Sambrook *et al*. (Molecular Cloning, A laboratory manual, Cold Spring Harbor Lab, Cold Spring Harbor, NY, 1989). The nucleotide sequence of VR2332 ORF4 in pABV270, determined on an automated DNA sequencer (Applied Biosystems), was identical to the published sequence (Murtaugh *et al*., Arch. Virol. 140; 1451-1460, 1995).

Subsequently, ORF4 of I-1102 and VR2332 were transferred to Semliki Forest virus expression vector pSFV1. pABV209 and pABV270 were digested with BamHI and HindIII (partially for pABV270), the ORF4 fragments were treated with Klenow polymerase (Pharmacia) to create blunt ends and these were ligated in the SmaI site of pSFV1, dephosphorylated with calf intestinal alkaline phosphatase (Pharmacia). Plasmids containing ORF4 of I-1102 (pABV265) and VR2332 (pABV271) in the correct orientation were further tested for expression of the GP_4 protein. In addition, four different chimeric ORF4 genes of I-1102 and VR2332 were made. The nucleotide sequence of ORF4 encoding amino acids 1 through 39 of the GP_4 protein of VR2332 was amplified from plasmid pABV270 with oligonucleotides PRRSV4 and PRRSV6. The obtained fragment was digested with BamHI and SacII. This fragment was ligated in pABV209 digested with BamHI and SacII to create an in frame fusion between amino acids 1 through 39 of the GP_4 protein of VR2332 and 40 through 183 of the GP_4 protein of I-1102 in pABV306. The nucleotide sequence of ORF4 encoding amino acids 1 through 75 of GP_4 of VR2332 was amplified with oligonucleotides PRRSV4 and PRRSV9 (see Table 2). This fragment was digested with KpnI and BamHI. The nucleotide sequence of ORF4 encoding amino acids 80 to 183 of the I-1102 GP_4 protein were amplified with PRRSV46 and PRRSV14 and the amplified fragment was digested with KpnI and BamHI. Both fragments were ligated together in pGEM-4Z digested with BamHI and HindIII, resulting in plasmid pABV308. In the same way a complementary construct was created in pABV314 consisting of the nucleotide sequence encoding amino acids 1 through 79 of the I-1102 GP_4 protein amplified with PRRSV13 and PRRSV57 ligated to a fragment encoding amino acids 76 through 178 of VR2332, which was amplified with PRRSV10 and PRRSV5, in pGEM-4Z. A fourth chimeric construct consisted of a fragment encoding amino acids 40 through 79 of the PRRSV GP_4 protein fused to fragments encoding amino acids 1 through

39 and amino acids 76 through 178 of the VR2332 GP_4 protein. This was achieved by ligating the BamHI/SacII ORF4 fragment of pABV270 and the SacII/HindIII ORF4 fragment of pABV314 in pGEM-4Z digested with BamHI and HindIII. This plasmid was designated pABV325. Plasmids pABV306, pABV308, pABV314, and pABV325 were checked for the correct sequence by oligonucleotide sequencing. The chimeric ORF4 genes were transferred from pABV306, pABV308, pABV314, and pABV325 to PSFVI, identical as described above for the ORF4 genes of VR2332 and PRRSV, resulting in pABV296, pABV305, pABV321, and pABV326, respectively (Fig. 3).

Two oligonucleotides located upstream (LV108;5' GGAGTGGTTAACCTCGTCAAGTATGGCCGGTAAAAACCAGAGCC 3') and downstream (LV112;5' CCATTCACCTGACTGTTTAATTAACTTGCACCCTGA 3') of ORF7 were used to amplify and clone the ORF7 gene in pGEM-T, resulting in pABV431. The sequences and position of these and other oligonucleotides used to amplify fragments of ORF7 are listed in Table 1. In addition, four different chimeric constructs were made by PCR-directed mutagenesis. The sequences coding for amino acids 25-26, 28-30 (site B; figure 3) were substituted for the corresponding sequences of the EAV N protein. This was accomplished by PCR amplification of ORF7 with LV108 and LV134 (5' TGGGGAATGGCCAGCCAGTCAATGACCTGTGCCGGATGTTTGGTGCAATGATAAAGTCC 3'). The mutated DNA fragment was introduced in pABV431 using the *Msc*I and *Pac*I site, which resulted in pABV455. The region of ORF7 encoding amino acids 51 to 67 was substituted for the corresponding region of LDV ORF7. pABV431 was digested with *EcoN*I and *Cla*I and ligated to a PCR fragment produced with primers LV98 (5' CCAGCAACCTAGGGGAGGACAGGCCAAAAAGAAAAAGCAGCCGAAGCTACATTTTCCCATG GCTGGTCCATCTGAC 3') and LV99 (5' CGTCTGGATCGATTGCAAGCAGAGGGAGCGTTCAGTCTGGGTGAGGACGTGCCGGAGGTCA GATGGACCAGCC 3') , digested with the same enzymes. This plasmid was designated pABV463. The region of ORF7 encoding amino acids 80 to 90 was substituted for the corresponding

region of the LDV ORF7 gene. The ORF7 gene of LV was mutated in a PCR with primers LV101 (5' GCTTGCAGGCGCCCGTGACGCTTTTCAATCAAGGCGGAGGACAGGCGTCGCTTTCATCCA3') and LV112. The obtained fragment was digested with *Nar*I and *Pac*I and ligated to pABV431 digested with *Cla*I and *Pac*I. This resulted in pABV453. Finally, the region encoding the C terminal part of the N protein (amino acids 111-128) was replaced for a sequence encoding the corresponding amino acids of the N protein of LDV. The ORF7 gene was amplified with primers LV108 and LV102 (5' ATGTCCCGGGCTAAGCGGCGGAGGAATTAGCAGAAGCGTTAATCAGGCGCTGTGTAGCAGC AACCGGCAG 3') and cloned in the pGEM-T vector, which resulted in pABV456. The wild type and mutated ORF7 genes were excised from pABV431, pABV453, pABV455, and pABV463 by digestion with *Pac*I (blunt ended) and *Hpa*I and from pABV456 by digestion with *Hpa*I and *Swa*I. These genes were subsequently inserted in the dephosphorylated *Sma*I site of Semliki forest virus expression vector pSFV1. Plasmids pABV470, pABV460, pABV462, pABV518 and pABV471 containing the respective ORF7 genes in the correct orientation were further tested for expression of the N protein. In vitro transcription and transfection of Semliki forest virus ORF7 RNA was as described above for the SFV-ORF4 constructs.

To clone the ORF4 genes of seven different European isolates macrophages were infected with NL1, NY2, DEN, FRA, SPA1, SPA2, and LUX, and RNA was isolated as described by Meulenberg *et al.* (1993). The ORF4 genes were amplified by means of RT-PCR with oligonucleotides PRRSV13 and PRRSV14, and cloned with BamHI and HindIII in pGEM-4Z. For each strain, the nucleotide sequence of ORF4 of two clones derived from two independent PCRs was determined. The protein sequences derived from the nucleotide sequence were aligned using the multiple sequence alignment program CLUSTAL of PCGene (Intelligenetics Tm).

In vitro transcription and transfection of SFV-ORF4 RNA.

pSFV1 plasmids containing different ORF4 constructs were linearized by digestion with SpeI and transcribed *in vitro*. The synthesized RNA was transfected to BHK-21 cells in 15 mm wells of twentyfour-well plates using lipofectin. Cells were fixed with ice-cold 50% (v/v) methanol/aceton and the GP_4 protein expressed by the different ORF4 constructs was stained with MAbs in the immunoperoxidase monolayer assay (IPMA). To analyze the ORF4 expression products by immunoprecipitation, 10^7 BH K-21 cells were transfected with 10 µg *in vitro* transcribed SFV-ORF4 RNA by electroporation. The electroporated cells were plated in three 35 mm wells of six-well plates and 18 h after transfection cells were labeled.

Pepscan method

A complete set of overlapping nonapeptides or dodecapeptides was synthesized from amino acids derived of the ORF4 or ORF7 sequence of PRRSV, as was determined previously (Meulenberg *et al.*, 1993). The synthesis of solid-phase peptides on polyethylene rods and immunoscreening with an enzyme-linked immunosorbent assay (ELISA) type of analysis were carried out according to established PEPSCAN procedures (Geysen *et al.*, PNAS, 81, 3998-4002, 1984).

RESULTS

We have previously described a panel of neutralizing MAbs that reacted with a 31 to 35 kDa protein of PRRSV, designated GP_4, and a panel of Mabs reactive with the N protein, by Western immunoblot analysis. GP_4 was shown to be a structural glycoprotein encoded by ORF4, N was shown to be the nucleocapsid protein encoded by ORF7. In immunoprecipitation experiments with GP_4 specific Mabs, the GP_4 protein derived from lysates of cells infected with PRRSV, migrated as a discrete band of 28 kDa together with a light smear of somewhat higher apparent molecular weight. The

MAbs immunoprecipitated a diffuse (glycosylated) GP_4 protein of about 31 kDa from the extracellular medium of PRRSV-infected but not from the extracellular medium of mock-infected cells.

Identification of the neutralizing domain in GP_4.

We have demonstrated earlier that MAbs specific for the GP_4 protein recognized I-1102 but not the US isolate VR2332 (van Nieuwstadt *et al.*, 1996). In order to identify the binding domain of the neutralizing MAbs in the GP_4 protein, we made fusion proteins of the GP_4 protein of I-1102 and VR2332. These proteins were expressed in the Semliki Forest virus expression system, developed by Liljeström *et al.* (Biotechnol, 9, 1356-1362, 1991). First, ORF4 of I-1102 was cloned in pSFV1 resulting in plasmid pABV265 (Fig. 1). RNA transcribed from pABV265 was transfected to BHK-21 cells and 24 h after transfection cells were positively stained with the panel of fifteen neutralizing MAbs. The MAbs did not react with BHK-21 cells transfected with pSFV1 -RNA. The recombinant GP_4 protein was immunoprecipitated with MAb 126.1 from L-[^{35}S]-methionine labeled BHK-21 cells transfected with pABV265 RNA. It had a similar size as the authentic GP, protein synthesized in CL2621 cells infected with I-1102 and also contained PNGaseF and EndoH sensitive N-glycans. The GP_4 protein of VR2332 was also cloned in pSFV1, but this protein was not recognized by the MAbs upon expression in BHK-21 cells (Fig. 1). To further localize the region in the GP_4 protein recognized by the MAbs, four chimeric genes of ORF4 of I-11022 and VR2332 were constructed in pSFV1 (Fig. 1). RNA transcribed from plasmids pABV296, pABV305, pABV321, and pABV326 was transfected to BHK-21 cells and the reactivity of the expressed proteins with the GP_4-specific MAbs was tested in IPMA. The reaction pattern of these fifteen MAbs was identical, and indicated that these MAbs were directed to a region of 40 amino acids in the GP_4 protein; The expression

product of pABV326, consisting of amino acids 40 through 79 derived from the GP_4 protein of isolate CNCM I-1102 and surrounded by sequences derived from the VR2332 GP_4 protein was still recognized by the panel of MAbs. To ensure that the different GP_4 proteins, especially those which were not recognized by the MAbs, were properly expressed in BHK21 cells, they were immunoprecipitated from lysates of BHK-21 cells that were transfected with RNA transcribed *in vitro* from plasmids pABV265, pABV271, pABV296, pABV305, pABV321, and pABV326. Immunoprecipitation was carried out with porcine anti-PRRSV serum 21, MAb 126.1, and anti-peptide sera 698 and 700. Serum 700 is directed against amino acids 106-122 of the PRRSV GP_4 protein of isolate CNCN I-1102, a sequence which is identical in the GP_4 protein of isolate ATCC-VR2332, apart from amino acid 121. Therefore all GP_4 proteins were immunoprecipitated with serum 700. They were indistinguishable in size, when analyzed by SDS-PAGE, except for the GP_4 proteins expressed by pABV305 and pABV271, which migrated slightly faster. This is most likely due to the deletion of 4 amino acids in the VR2332 sequence relative to the I-1102 sequence, between amino acids 62-64 (Fig. 3). The complete set of GP_4-specific MAbs recognized the GP_4 proteins expressed from pABV265, pABV296, pABV321, pABV326, but not those expressed from pABV305 and pABV271, which confirmed the results obtained by IPMA (Fig. 3). Serum 698 had the same reaction profile as the MAbs. Serum 698 is directed against amino acids 62 to 77 of GP_4 of PRRSV, which are located within the now identified neutralization domain of the GP_4 protein. This region is highly heterogeneous in VR2332 ORF4, and therefore the expression products containing the VR2332 sequence in this region were not recognized by this serum. However, neutralizing polyclonal pig sera recognize the I-1102 GP_4 protein and the chimeric GP_4 proteins and the VR2332 GP_4 protein, indicating that in porcine anti-PRRSV sera a variety of neutralizing antibodies that are directed against

the neutralizing site formed by amino acids 40 to 79 of the GP_4 protein are present.

Pepscan of the ORF4 and ORF7 protein

Since the fifteen MAbs reactive with the ORF4 protein all reacted with the GP_4 protein in western blot analysis, they were expected to recognize a linear epitope in a region spanning amino acids 40 through 79 of GP_4 of isolate I-1102. To further map the binding region of the MAbs, a PEPSCAN analysis was performed using overlapping nonapeptides oe dodecapeptides in this region. Peptides were considered to represent antigenic sites if peaks in such a set reproducibly amounted to more than twice the background. MAbs 122-29, 122-30, 122-66, 122-71, 130-7, 138-28 reacted positively with one specific antigenic site consisting of amino acids 59 through 67 (SAAQEKISF) (Fig. 2). MAb 122-12 reacted only weakly to this antigenic site, whereas the remaining 7 MAbs were negative in the PEPSCAN analysis. Polyclonal pig sera also identified this site in PEPSCAN. Neutralizing serum 21, taken at week 6 after infection of pig 21 with PRRSV reacted strongly and broadly with the site and its flanking regions. In addition, neutralizing polyclonal pig sera (va12 and va14), taken at 54 days after vaccination with PRV-ORF4 vector virus and at slaughter at 30 days after challenge at day 54 with PRRSV, reacted strongly and more broadly with the neutralization site identified in PEPSCAN.

In isolate I-1102, the core sequence of the neutralization site comprises the aa sequence SAAQEKISF located from aa position 59-67. In other isolates the core sequence can be found at or around the corresponding aa position, which is an amino acid sequence corresponding to a neutralization site of protein GP4, comprising for example sequences such as SAAQEEISF, or STAQENISF or STAQENIPF or SEESQSVT or SASEAIR or SASEAFR or PAPEAFR or PAPEAIR or SAFETFR or STSEAFR, but it is to be expected that other isolates of PRRSV have corresponding but slightly differing core sequences of the

neutralization site located at or around the aa position corresponding to aa 59-67 of the ORF 4 amino acid sequence of the I-1102 isolate of PRRSV. Also, artificial changes that maintain the antigenicity and thus functionality of the above core sequences can easily be introduced by the average expert skilled in the art of peptide design and synthesis. Also, as is clearly demonstrated by the much broader reactivity in PEPSCAN of the neutralizing polyclonal sera, aa sequences comprising aa core sequences and aa sequences flanking the core sequences of the various PRRSV isolates in addition constitute the neutralization site on the ORF4 protein of PRRSV. Especially sequences located at positions corresponding to about aa 40 to 79 constitute the neutralization site (Fig 1). Again, artificial changes that maintain the antigenicity and thus functionality of the above antigenic sites can easily be introduced by the average expert skilled in the art of peptide design and synthesis. Also, considering the broad reactivity of the polyclonal neutralizing sera va12 and va14 (Fig 2), aa sequences located at positions corresponding from about aa 52 to 75 more specifically constitute a broadly reactive neutralization site.

The Mabs directed against the ORF7 protein reacted in four different groups in PEPSCAN, group A(4), B(2), C(3) and D(1). Group 1(D) (in which among others Mabs 122.17, 130.3, 130.4, 131.7, WBE1, WBE4, WBE6, SDOW17 and comprising conserved and non-conserved reactive sites) reacted with a conformational epitope not detectable in PEPSCAN. Group 2(B) (in which among others 125.1, 126.9, NS95 and NS99 and reactive with all isolates of PRRSV tested, thus identifying a conserved antigenic site) identifies a core sequence VNQLCQLLGA (found in isolate I-1102 from aa position 22 to about 32) or VNQLCQMLGK. Group 3(C) (in which among others Mab 126.15 and mainly reactive with strains of PRRSV isolated in Europe, thus identifying a differentiating antigenic site) identifies a core sequence PRGGQAKKKK (found in isolate I-1102 from aa position 41 to about 50) or PRGGQAKRKK or PRGGQAKKRK or

GPGKKNKKKN or GPGKKNKKKT or GPGKKNRKKN or GPGKKFKKKN or GPGKKIKKKN or GPGQINKKIN. Group 4(A) (in which among others Mab 138.22 and mainly reactive with strains of PRRSV isolated in Europe, thus identifying a differentiating antigenic site) identifies a core sequence MAGKNQSQKK (found in isolate I-1102 from aa position 1 to about 10) or MPNNNGKQTE or MPNNNGKQPK or MPNNNGKQQK or MPNNNGKQQN or MPNNNGKQQK or MPNNNGRQQK. Also, artificial changes that maintain the antigenicity and thus functionality of the above antigenic sites in the N protein can easily be introduced by the average expert skilled in the art of peptide design and synthesis. Although group 1 does not constitute linear epitopes, comparison of PRRSV aa sequences with LDV sequences shows that conformational epitopes (which vary greatly among the various isolates) can be found at positions corresponding to those found in isolate I-1102 from aa position 51 to about 68 (in isolate I-1102 aa sequence PKPHFPLAAEDDIRHHL) or from 79 to about 90 (in isolate I-1102 aa sequence SIQTAFNQGAGT) or from 111 to 124 (in isolate I-1102 aa sequence HTVRLIRVTSTSAS). Also, artificial changes that maintain the antigenicity and thus functionality of the above conformative epitope sites in the N protein can easily be introduced by the average expert skilled in the art of peptide design and synthesis, especially with information gathered by sequence comparison of PRRSV isolates, and by comparison with N protein sequences of other Arteriviridae. This was determined in expressing chimeric LDV/PRRSV ORF7 proteins in the SFV expression system (done as above for ORF4) and determining their reactivity with Mabs from group 1.

Chimeric N proteins

Domain D was further mapped with constructs of ORF7 expressing chimeric N proteins. Since 6 out of 10 MAbs directed against domain D recognized both European and North American isolates of PRRSV, the regions which were most conserved between the N protein of LV and the North American

prototype VR2332 (Fig. 4) were mutated. The nucleotide sequence coding for amino acids 51 to 67, 80 to 90, and 111 to 128 was substituted for a sequence that codes for the corresponding amino acids of LDV (Fig. 4). For completion, site B (amino acids 25-30) that is also conserved in European and North American isolates, was mutated. Since the amino acid sequence of the LV N protein was very similar to that of the LDV N protein in site B, this region of the LV N protein was substituted for a region encoding the corresponding amino acids of the EAV N protein (Fig. 4). When the mutated and wild type N proteins were expressed in BHK-21 cells using the Semliki forest virus expression system, and they were tested with the N-specific MAbs in IPMA, the D-specific MAbs reacted identical (Table 1). Their binding was disrupted by mutations between amino acids 51-67 and 80-90, but not by mutations between amino acids 111-128 or amino acids 25-30 (site B). As was expected, the N proteins with LDV sequences between amino acids 51-67 and 80-90 were still stained by MAbs directed against sites A, B, and C. However, the number of cells that were stained and the brightness of this staining was less than that observed for the wildtype N protein and the N proteins mutated in amino acids 25-30 (site B) or in amino acids 111-128 (Table 1). This was most likely due to a lower expression of the N proteins containing mutations between amino acids 51-67 or 80-90, since a lower yield of these mutant N proteins compared to the other N proteins was also obtained when equal amounts of transcripts were translated in vitro (data not shown). As was expected, the N protein that contained EAV sequences in site B was not recognized by MAbs mapped to site B (by pepscan analysis), but was still recognized by MAbs that mapped to sites A, C, or domain D. These data indicate that the epitopes mapped to domain D are conformation-dependent and consist (partially) of amino acids 51-67 and 80-90.

Sequence analysis of the GP_4 protein of different PRRSV strains.

To analyze whether the major antigenic neutralization site, recognized by the GP_4-specific antibodies, was conserved among different PRRSV isolates, the reactivity and neutralizing activity of the MAbs was further tested on seven different European strains. The results indicated that these MAbs recognized and neutralized another Dutch strain NL1 and an English strain NY, but not Danish isolate DEN, two Spanish strains SPA1 and SPA2, a French isolate FRA, and LUX isolated in Luxembourg. Therefore we were interested in the amino acid sequence, in the region of the neutralization site of the GP_4 protein of these isolates. The ORF4 genes were cloned by means of RT-PCR using primers derived from the PRRSV sequence and the nucleotide sequence was determined. The amino acid sequence of the GP_4 protein of the different isolates derived from this nucleotide sequence were 86 to 97% identical with that of I-1102. The alignment of these amino acid sequences showed that the neutralization site (amino acids 40 through 79) is much more divergent than the remaining part of the protein. In this region, especially the amino acid sequences of strains DAN, SPA1, SPA2 and FRA are different. This is in line with the finding that these strains are not neutralized by the I-1102 specific MAbs and further confirms that this site is not highly conserved among European isolates. Another region of higher heterogeneity was observed in the N-terminal part of the GP_4 protein. Comparison of the amino acid sequence of the PRRSV GP_4 protein and that of VR2332 and other North American strains shows that the latter are also heterogenous in the neutralization site of the protein. Alignment of the amino acid sequences results in the introduction of a gap in the neutralization site of the North American isolates (Fig. 3), which is in agreement with the observation that none of these isolates are recognized by the MAbs. Overall, a higher diversity was observed among the

sequences of the American isolates than among the sequences of the European isolates.

This is in line with the features characteristic for typical viral envelope, identified e.g. in the amino acid sequence of GP_4.

The potential of the neutralization site for vaccine development is of great importance in view of the heterogenicity of the neutralization site. Comparison of the amino acid sequence of the GP_4 proteins of different European strains indicated that the neutralization site was much more variable than other parts of the protein, suggesting that this site is susceptible to immunoselection. Comparison of the neutralization site sequences of European and North American strains displayed a gap of 4 amino acids in the North American sequences with respect to the European, further illustrating the large amino acid variability of the now identified neutralization site of PRRSV.

The neutralization site in the GP_4 protein described here, is the first site identified for Lelystad virus. For two other arteriviruses, EAV and LDV, the neutralizing MAbs that were isolated, were all directed against the G_1/VP3 protein encoded by ORF5 (Deregt *et al*, 1994; Glaser *et al*, 1995; Balasuriya *et al*, 1995; Harty and Plagemann, 1988). Using neutralization-escape mutants, the neutralization site of EAV was mapped to specific amino acids in the ectodomain of G_1.

Similar sequence comparisons were done for the ORF7 protein of PRRSV (fig. 4) further illustrating the large amino acid variability of the now identified antigenically conserved site and non-conserved sites of PRRSV. In this work we have identified four distinct antigenic sites in the N protein of PRRSV. Three sites, designated A, B, and C contain linear epitopes and these were mapped between amino acids 2-12, 25-30, and 40-46, respectively.In contrast, the fourth site, designated domain D, contains conformation-dependent epitopes that are (partially) composed of amino acids 51-67

and 80-90. Sites A and C contain epitopes that are conserved in European but not in North American isolates of PRRSV, site B contains epitopes that are conserved in European and North American isolates of PRRSV, whereas site D contains both epitopes that are conserved and not conserved in European and North American isolates of PRRSV. The conserved sites in the N protein described here, are of great importance in the development of diagnostic tests aimed at unequivocal diagnosis of PRRSV infections, these tests should avoid employing non-conserved sites thereby avoiding false-negative results. In addition, knowledge about the various non-conserved sites is highly valuable in the development of differentiating tests that can e.g. discriminate vaccinated pigs from pigs infected with wild type isolates of PRRSV.

Legends.

Fig. 1. Schematic diagram of G proteins expressed in pSFV1 and their reactivity with GP_4-specific MAbs. The names of the plasmids containing the different ORF4 genes are indicated. Open bars represent the amino acid sequences derived from the G protein encoded by ORF4 of PRRSV, black bars represent amino acid sequences derived from the G protein encoded by ORF4 of VR2332. The numbers of the amino acids are indicated above the bars. The genes were first inserted in PGEM-4Z and then transferred to pSFV1, as described in detail in the Materials and methods section. The complete set of 14 GP_4-specific MAbs reacted identically with the different constructs in IPMA and the reactivity is indicated as positive (+) and negative (-).

Fig. 2. Pepscan analysis of GP_4-specific MAbs and polyclonal sera with overlapping 12-mer peptides covering residues 25 to 94 of GP_4. The scan of MAbs 130.7 and MAb 138.28 which recognize four consecutive peptides is shown (2A). Five other MAbs (122.29, 122.30, 122.66, 122.71 and 138.28) exhibited similar specificity. The scans of polyclonal sera from two pigs before immunization (va12-0 and va14-0), after immunization with a pseudorabies virus vector expressing ORF4 (va12-54 and va14-54) and after subsequent challenge with PRRSV (va12-s1 and va14-s1) are shown (2A and 2B), and the scan of polyvalent porcine anti-LV serum 21 (va21) is shown (2D). The amino acid sequence of the reactive peptides is shown with the core of common residues boxed.

Fig. 3. Alignment of amino acid sequences of GP_4 (A) and N (B) proteins of various PRRSV strains. Only the amino acids which differ from the I-1102 sequence are shown. The core peptide sequences recognized by MAbs and/or polyclonal sera in pepscan analysis are underlined.

Fig. 4. Location of antigenic binding sites in the N protein sequence and comparison of the N protein sequence with those of North American strain VR2332 and LDV. Antigenic sites A, B, C, and domain D are shown in shadow. Sites A, B, and C were identified in pepscan analysis, site D was identified by construction of chimeric N proteins. The amino acid sequences of LV that were substituted for the corresponding amino acid sequences of LDV in order to map domain D are underlined. The amino acids of the N protein of EAV that were inserted between amino acids 25-30 to mutate site B are shown below the LDV sequence. Identical amino acids are connected with vertical bars.

Table 1. Staining of chimeric N proteins expressed by Semliki forest virus in BHK-21 cells in IPMA

Plasmid	Mutation	Staining with MAbs in IPMA									
		138.22	125.1/126.9/NS95/NS99			126.15	122.15/130.2/130.4/131.7/131.9/WBE1/WBE4/WBE5/WBE6/SDOW17				
		A	B			C	D				
pABV470	-	+++	+++			++	+++				
pABV462	25-30	+++	-			++	+++				
pABV518	51-67	++	++			+	-				
pABV460	80-90	++	++			+	-				
pABV471	111-124	+++	+++			++	+++				

Table 2. Sequence of primers used in PCR to clone the ORF4 genes of LV and VR2332 and chimeric ORF-4 genes in plasmid vectors pGEM-4Z and pSFV1

Name	Sequence[a]	Incorporated restriction site
LV13	5' GGCAATT*GGATCC*ATTTGGA 3'	*Bam*HI
LV14	5' AGAAGC*AAGCTT*GCGGAGTC 3'	*Hind*III
LV46	5'GCCGTC*GGTACC*CCTCAGTACAT 3'	*Kpn*I
LV57	5'ATGTACTGAGG*GGTACC*GACGGC 3'	*Kpn*I
PRRSV4	5' GGCAATT*GGATCC*ACCTAGAATGGC 3'	*Bam*HI
PRRSV5	5' GCGAGC*AAGCTT*CCGCGGTCAAGCATTTCT 3'	*Hind*III
PRRSV6	5'CTTGCCG*CCGCGG*TGGTGTTG 3'	*Sac*II
PRRSV9	5' ACAGCT*GGTACC*TATCGCCGTACGGCACTGA 3'	*Kpn*I
PRRSV10	5' GCGATAGG*TACC*CCTGTGTATGTTACCAT 3'	*Kpn*I

[a] The underlined nucleotides in these primers are mutated with respect to the original sequence to create restriction sites or overhanging sequences or to avoid long stretches of one particular nucleotide. The restriction sites in the primers are shown in italics.

CLAIMS

1. A peptide eliciting neutralizing antibodies comprising an amino acid sequence of at least 7 to 40 amino acid residues derived from an amino acid sequence corresponding to a neutralization site of protein GP4 of PRRSV.

2. A peptide eliciting neutralizing antibodies according to claim 1 comprising an amino acid sequence wherein amino acid residues have been replaced conventionally or under guidance of replacement mapping.

3. A peptide according to claim 1 or 2 wherein said neutralization site comprises the amino acid residues located at about amino acid position 40 to 79 of protein GP4 of PRRSV isolate I-1102.

4. A peptide according to any of claims 1 to 3 wherein said neutralization site comprises the amino acid residues located at about amino acid position 52 to 75 of protein GP4 of PRRSV isolate I-1102.

5. A peptide according to any of claims 1 to 4 wherein said neutralization site comprises the amino acid residues located at about amino acid position 59 to 67 of protein GP4 of PRRSV isolate I-1102.

6. A peptide according to any of claims 1 to 5 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of SAAQEKISF, SAAQEEISF, STAQENISF, STAQENIPF, SEESQSVT, SASEAIR, SASEAFR, PAPEAFR, PAPEAIR, SAFETFR and STSEAFR.

7. A peptide eliciting antibodies which react with at least two different PRRSV isolates comprising an amino acid sequence of at least about 5 to about 15 amino acid residues derived from an amino acid sequence corresponding to a conserved site of protein N of PRRSV.

8. A peptide eliciting antibodies which react with at least two different PRRSV isolates according to claim 7 comprising an amino acid sequence wherein amino acid residues have been

replaced conventionally or under guidance of replacement mapping.

9. A peptide according to claim 7 or 8 wherein said conserved site comprises the amino acid residues located at about amino acid position 22 to 32 of protein N of PRRSV isolate I-1102.

10. A peptide according to any of claims 7 to 9 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of VNQLCQLLGA and VNQLCQMLGK.

11. A peptide eliciting antibodies which are capable of distinguishing between at least two different PRRSV isolates comprising an amino acid sequence of at least 5 to 15 amino acid residues derived from an amino acid sequence corresponding to a non-conserved and differentiating site of protein N of PRRSV.

12. A peptide eliciting antibodies which are capable of distinguishing between at least two different PRRSV isolates according to claim 11 comprising an amino acid sequence wherein amino acid residues have been replaced conventionally or under guidance of replacement mapping.

13. A peptide according to claim 11 or 12 wherein said non-conserved site comprises the amino acid residues located at about amino acid position 41 to 50 of protein N of PRRSV isolate I-1102.

14. A peptide according to any of claims 11-13 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of PRGGQAKKKK, PRGGQAKRKK, PRGGQAKKRK, GPGKKNKKKN, GPGKKNKKKT, GPGKKNRKKN, GPGKKFKKKN, GPGKKIKKKN and GPGQINKKIN.

15. A peptide according to claim 11 wherein said non-conserved site comprises the amino acid residues located at about amino acid position 1 to 10 of protein N of PRRSV isolate I-1102.

16. A peptide according to claim 11, 12 or 15 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of MAGKNQSQKK, MPNNNGKQTE, MPNNNGKQPK, MPNNNGKQQK, MPNNNGKQQN, MPNNNGKQQK and MPNNNGRQQK.

17. A peptide eliciting antibodies directed against a conformational epitope site or protein N of PRRSV, said peptide having a length of from 5 to 15 amino acid residues, said amino acid residues corresponding to a conformational stretch located from about amino acid position 68 to 79 or from 79 to 90 or from 111 to 124 of protein N of Lelystad virus isolate I-1102.

18. A peptide according to claim 17 wherein said amino acid sequence comprises an amino acid sequence selected from the group consisting of PKPHFPLAAEDDIRHHL, SIQTAFNQGAGT and HTVRLIRVTSTSAS.

19. An immunogenic composition for inducing neutralizing antibodies corresponding to a neutralization site of protein GP4 of Lelystad virus comprising at least one peptide according to any of claims 1 to 6.

20. A vaccine for the prophylaxis of PRRS infections comprising a peptide according to any of claims 1 to 6 or an immunogenic composition of claim 19, together with a suitable adjuvant or carrier for administration to an animal.

21. A synthetic antibody reactive with a peptide according to any of claims 1-18.

22. A diagnostic test kit for the detection or identification of antibodies directed against a PRRSV isolate comprising at least one peptide of any of claim 1 to 18 together with suitable means for detection.

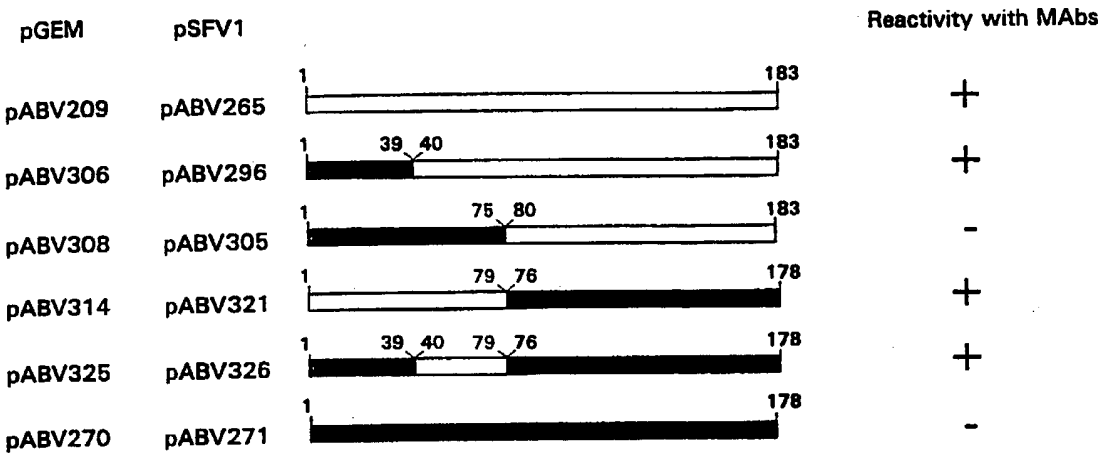
23. A diagnostic test kit for the detection or identification of antibodies directed against or antigen derived from a PRRSV isolate comprising an antibody according to claim 21.

24. Use of a vaccine according to claim 20 or a diagnostic test or kit according to claim 22 or 23 to reduce the occurrence of PRRS in a pig herd.

25. Use of a diagnostic test kit according to claim 22 or 23 to test for the occurrence of PRRS in a pig or pig herd.

26. Use of a vaccine according to claim 20 or a diagnostic test or kit according to claim 22 or 23 in eradication programs to reduce or terminate the occurrence of PRRS in a pig herd.

27. A method for testing for the occurence of PRRS in a pig or a pig herd comprising the use of a diagnostic test kit according to claim 22 or 24.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+
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75
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-
pABV314
pABV321
1
79
76
178
+
pABV325
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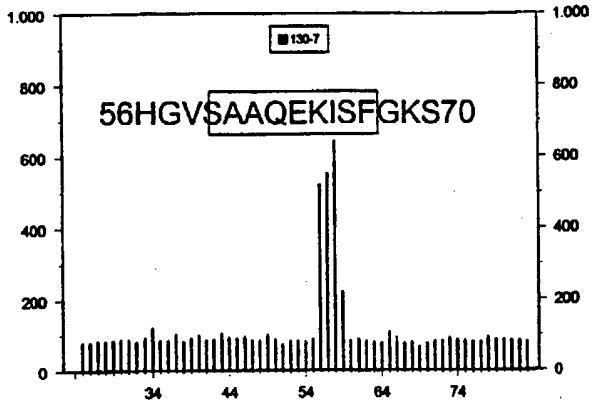
Fig. 1

Fig.2A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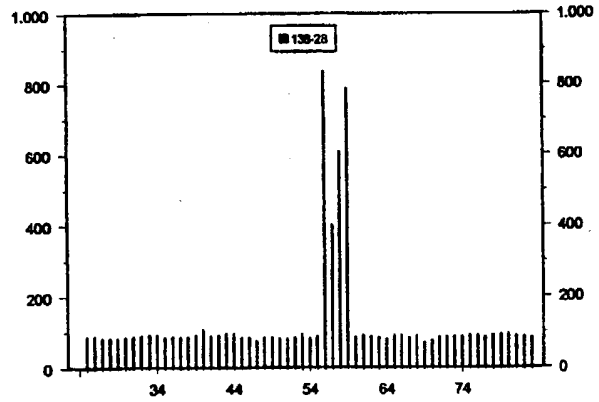
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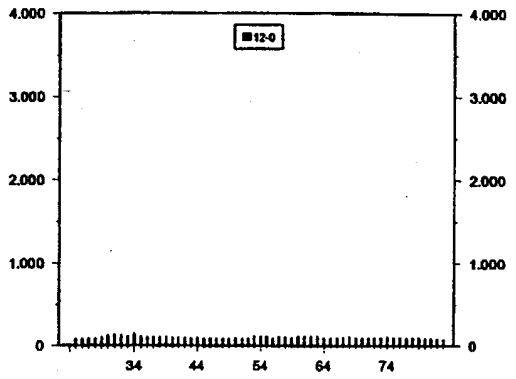
Fig.2B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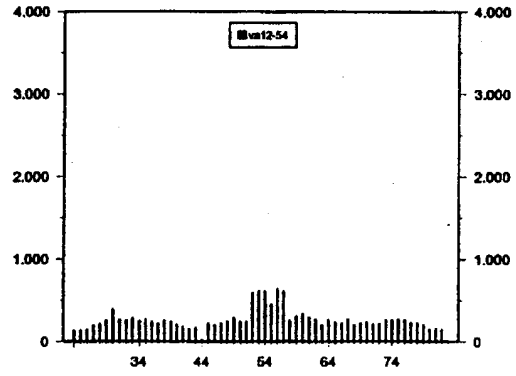
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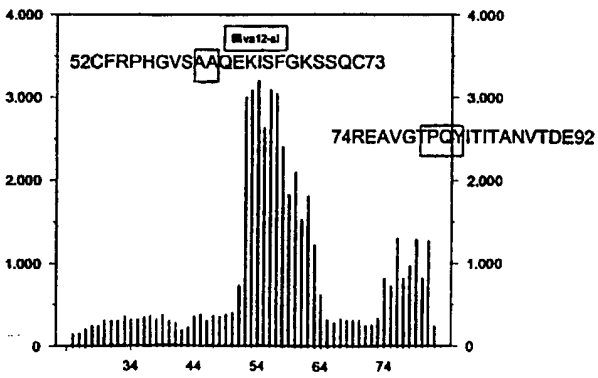
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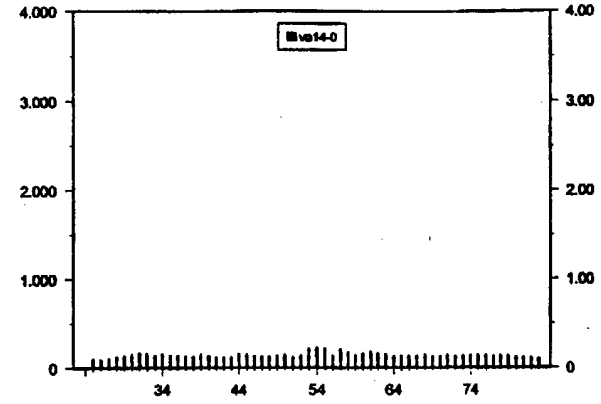
44
54
64
74

Fig.2C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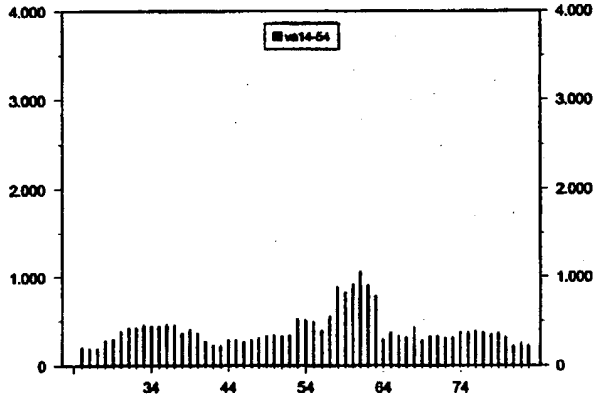
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0
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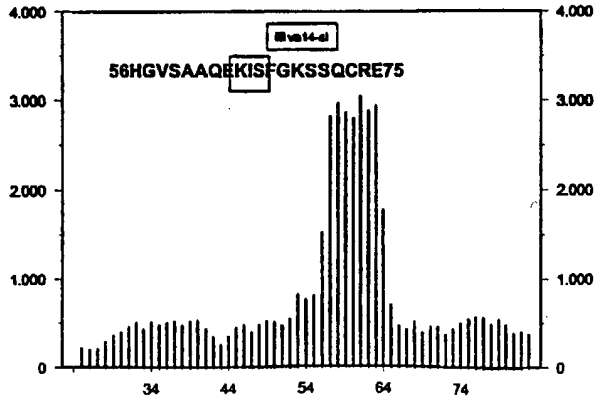
44
54
64
74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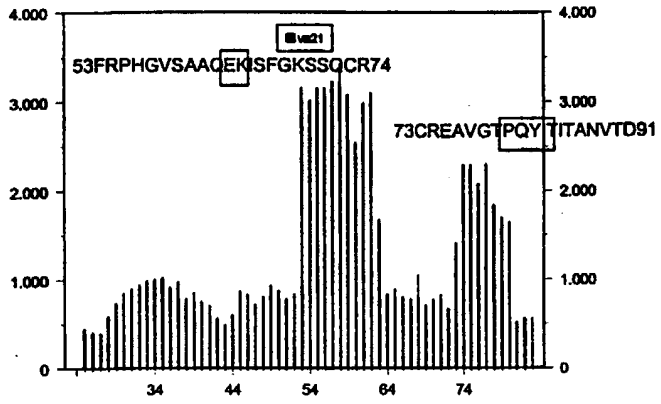
64
74

Fig.2D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34
44
54
64
74
53FRPHGVSAAQEKISFGKSSQCR74
73CREAVGTPQYTITANVTD91

Fig. 3A Alignment of amino acids of GP_4 of PRRSV isolates

```
I-1102-4     MAAATLFFLAGAQHIMVSEAFACKPCFSTHLSDIETNTTAAAGFMVLQDI     50
NL1-4                                                               50
NY2-4              L                                                50
PRRSV10-4          L                                                50
LUX-4              L                                  K             50
SPA1-4           I     S   F                         K           N  50
FRA-4            I   L     F                         K           N  50
DEN-4            I   L     L                         K              50
VR2332-4       SSL   LVV FKCLL  Q        SS A  K       S A         50
IA1-4           SL   L V FKCLL  Q        SS A  K       S A   E     50
KS1-4           SL   LMV FKCLL  Q        SS A  K       SSV         50
MN1-4           SL   LMV FKCLL  Q        SS A  K       S A         50
IA6-4           SL   L V FKCLL  Q        SS A  K       S A         50
IL1-4           SL   L V FKCFV  Q        SS A  K       S A         50
NE1-4          SSL   LMV FKCLL  Q        SS A  K       N A   E     50
SG1-4           SL   L V FKCLL  Q    NQ  SS  E K       G A   E     50
VR2385-4        SL   L V FKCLL  Q        SS    K       G A         50
KY1-4           SL   LMV FKCLL  Q        SS A  K       S A         50
MO1-4           SL   LMV FERLL  Q        SS A  K       G A   E     50
                **.. **.... . ..**.****..***. *..*.*******.  ***.*

I-1102-4     NCFRPHGVSAAQEKISFGKSSQCREAVGTPQYITITANVTDESYLYNADL    100
NL1-4                                                              100
NY2-4                                                              100
PRRSV10-4                                                          100
LUX-4          L   R       E                                       100
SPA1-4         L       T   N P   P        I                       100
FRA-4          L       T   N     P        I                       100
DEN-4        T A     A EESQSVT N P        I              H        100
VR2332-4     S L HRDSASE----AIR IP   T I   V V         N  HSS      96
IA-4         S L HRNSASE----AIR VP   T I   V           N  HSS      96
KS1-4        S L HRNSASE----AIR VP Y T I   V           N  HSS      96
MN1-4        S L HRNSASE----AIR IP   A I   V           N  HSS      96
IA6-4        S L HRDSASE----AIR  P   T I   V           N  HSS      96
IL1-4        S L HRDSASE----AIR IP   T I   V           N  HSS      96
NE1-4        S L HRNPAPE----A R IP   T I   V           N  HSS      96
SG1-4        S L HRNPAPE----AIR VP   T I   V  SV       N  HSS      96
VR2385-4     S L HRNSASE----AIR VP   T I   V   V       N  HSS      96
KY1-4        S L HRDSAFE----T R VP   T I   V   V       N  HSS      96
MO1-4        S L HRDSTSE----AFR VP   T I   V   V       N  HSS      96
             .* *..  . .    .. * .*.*.*.*.* *...********.**...**
```

Fig. 3A continued

```
I-1102-4     LMLSACLFYASEMSEKGFKVIFGNVSGVVSACVNFTDYVAHVTQHTQQHH   150
NL1-4                                                             150
NY2-4                                                             150
PRRSV10-4                                                         150
LUX-4                                                             150
SPA1-4                                                            150
FRA-4                                                             150
DEN-4                                                             150
VR2332-4         S               V       I AV      S  Q  KEF  -RS  145
IA1-4            S             E V       I AV      S  Q  REF  -RS  145
KS1-4            S             E V       I AV      S  Q  REF  -RS  145
MN1-4            S             E V       I AV      S  Q  REF  -RS  145
IA6-4            S               V       IAAV      S  Q  KES  -RS  145
IL1-4            S             E V       I AV      S  Q  REF  -RS  145
NE1-4            S               V       I AV      S  Q  KEF  -RS  145
SG1-4            S               V       I AV      S  Q  KEF  -RS  145
VR2385-4         S               V       I AV      S  Q  KEF  -RS  145
KY1-4            S               V         AV      S  Q IKEF  -PS  145
MO1-4            S               V         AV      S  Q IKEF  -RS  145
                 ****.*************.*.******....******.**.*. . ** .

I-1102-4     LVIDHIRLLHFLTPSAMRWATTIACLFAILLAI                 183
NL1-4                                                          183
NY2-4                                                          183
PRRSV10-4                                                      183
LUX-4                                                          183
SPA1-4                      T                                  183
FRA-4                       T                                  183
DEN-4         A                                                183
VR2332-4       V  V      M  ET      VL                         178
IA1-4         MV  V      M  ET      VL                         178
KS1-4         MV  V      M  ET      VL                         178
MN1-4         MV  V      M  ET      FL   G                     178
IA6-4          V  V      M  ET      VL                         178
IL1-4          V  V      M  ET      VL                         178
NE1-4          V  V      M  ET      VL F                       178
SG1-4          V  V      M  ET      VL                         178
VR2385-4       V  V      M  ET      VL    T                    178
KY1-4          V  V F    I  ET      VL   G   ---               175
MO1-4          V  V      I  ET      FL   G   ---               175
               * .**.*.***.**..***** .* * .**
```

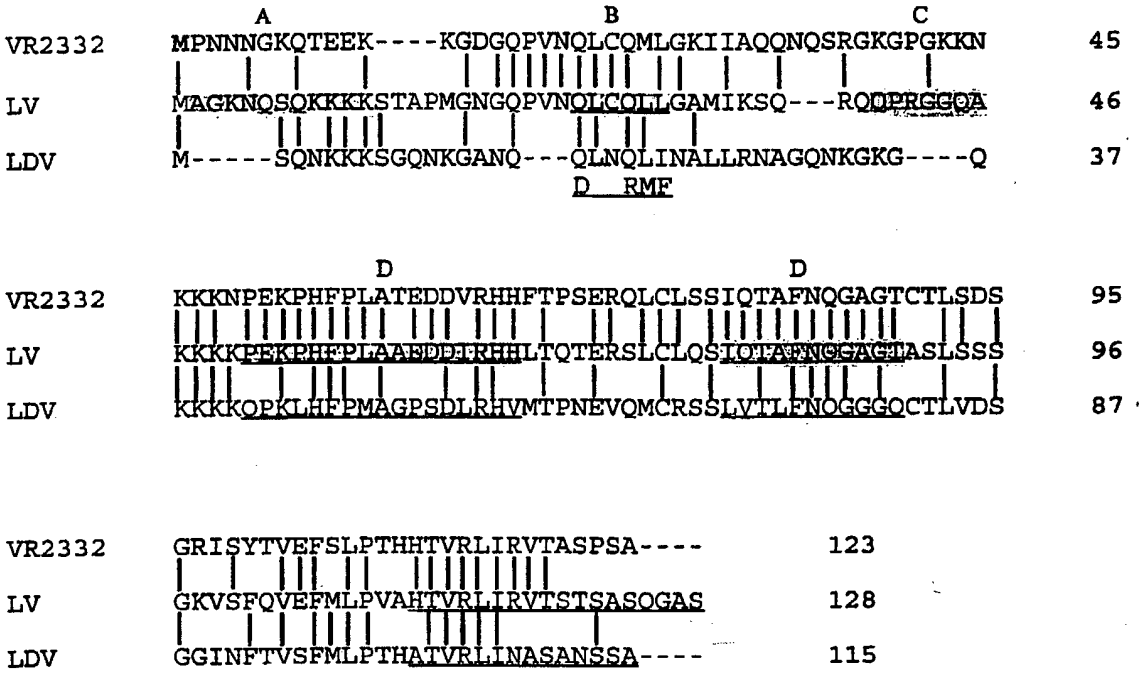
Fig. 3B Alignment of amino acid sequences of N of PRRSV isolates

```
I-1102-7      MAGKNQSQKKKKSTAPMGNGQPVNQLCQLLGAMIKSQ---RQQPRGGQAK    47
PRRSV10-7                                                        47
VR2332-7       PNN GK TEE ----K D          M  KI AQ NQS GKGP KKN    46
NE1-7          PNN GK TEE ----R D          M  KI AQ NQS GKGP KKN    46
IL1-7          PNN GK P E ----K D          M  KI AQ NQS GKGP KKN    46
IA1-7          PNN GK Q   ----K D          M  KI AQ NQS GKGP KKNR   46
IA6-7          PNN GK QN  ----K D          M  KI AQ NQS GKGP KKN    46
KS1-7          PHN GK Q R ----K D          M  KI AQ NQS GKGP KKF    46
MN1-7          PNN GK Q R ----K D          M  KI AQ NQS GKGP KKN    46
SG1-7          PNN GR Q   ----K DC         M  KI AQ NQS GKGP KKN    46
MO1-7          PNN GR Q   ----K D          M  KI AQ NQS GKGP KKI    46
KY1-7          PHN GR Q   ----K D          M  KI AQ NQS GKGP QIN    46
ISU3927-7      PNN GK Q   ----K D          M  KI AQ NQS GKGP KKN    46
ISU55-7        PNN GK Q   ----K D          M  KI AQ NQS GKGP KKN    46
ISU1894-7      PNN GK Q R ----K D          M  KI AQ NQS GKGP KKN    46
VR2385-7       PNNTGK Q R ----K D          M  KI AH NQS GKGP KKN    46
IAF-7          PNN GR Q   ----K D          M  KI AQ NQS GKGP KKN    46
               *. .. .* . *    .*. ********.** .*  *   * . .*   .

I-1102-7      KKKPEKPHFPLAAEDDIRHHLTQTERSLCLQSIQTAFNQGAGTASLSSSG    97
PRRSV10-7                                                   P     97
VR2332-7         N         T   V   F PS  Q   S            CT  D    96
NE1-7            T         T   V   F PS  Q   S            CT  D    96
IL1-7            N         T   V   F PS  Q   S            CT  D    96
IA1-7            N         T   V   F PS  Q   S            CI  D    96
IA6-7            N         T   V   F PS  Q   S            CT  D    96
KS1-7            N         T   V   F PS  Q   S            CT  D    96
MN1-7            N         T   V   F PS  Q   S           ICI  D    96
SG1-7            N         T V V   F PS  Q   S            CT  D    96
MO1-7            N        SI FV    F PS  Q   S            CT  D    96
KY1-7           IN V  YS  VT   V   F PS  Q   S            CT  D    96
ISU3927-7        N         T   V   F PS  Q   S            CI  D    96
ISU55-7          N         T   V   F SG  Q   S            CT  D    96
ISU1894-7        N         T   V   F PS  Q   S            CT  D    96
VR2385-7         N         T   V   F PS  Q   S            CT  D    96
IAF-7            N         T   V   F PS  Q   S            CT  D    96
               * .* **. **  *  .***.*..** *** ***********.  **.**

I-1102-7      KVSFQVEFMLPVAHTVRLIRVTSTSASQGAS   128
PRRSV10-7                                      128
VR2332-7      RI YT   S  TH        ASPSA----   123
NE1-7         RI YT   S  TH        ASPSA----   123
IL1-7         RI YT   S  TH        APSSA----   123
IA1-7         RI YT   S  TH        ASPSA----   123
IA6-7         RI YT   S  TH        APPSA----   123
KS1-7         RI YT   S  TH        ASPSA----   123
MN1-7         RI YT   S  TH        VSPSA----   123
SG1-7         RI YT   S  TH        ASPSA----   123
MO1-7         RI YT   S  TH        ASPSA----   123
KY1-7         RI YT   S  TH        ASPSA----   123
ISU3927-7     RI YT   S  TH        APPSA----   123
ISU55-7       RI YT   S  TH        APPSA----   123
ISU1894-7     RI YT   S  TH        ASPSA----   123
VR2385-7      RI YT   S  TH        ASPSA----   123
IAF-7         RI YA   S  TH        ASPSA----   123
              ..*. *** **. ********* ....
```

Fig. 4


A
B
C
VR2332 MPNNNGKQTEEK----KGDGQPVNQLCQMLGKIIAQQNQSRGKGPGKKN 45
LV MAGKNQSQKKKKSTAPMGNGQPVNQLCQLLGAMIKSQ---RQQPRGGQA 46
LDV M-----SQNKKKSGQNKGANQ---QLNQLINALLRNAGQNKGKG----Q 37
D RMF
D
D
VR2332 KKKNPEKPHFPLATEDDVRHHFTPSERQLCLSSIQTAFNQGAGTCTLSDS 95
LV KKKKPEKPHFPLAAEDDIRHHLTQTERSLCLQSIQTAFNQGAGTASLSSS 96
LDV KKKKQPKLHFPMAGPSDLRHVMTPNEVQMCRSSLVTLFNQGGGQCTLVDS 87
VR2332 GRISYTVEFSLPTHHTVRLIRVTASPSA---- 123
LV GKVSFQVEFMLPVAHTVRLIRVTSTSASQGAS 128
LDV GGINFTVSFMLPTHATVRLINASANSSA---- 115

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/NL 98/00251

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/08 C07K16/10 A61K39/12 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 04010 A (UNIV MINNESOTA) 15 February 1996 see claims; examples 12,13	1,7,11, 17,19, 20,24-26
X	GB 2 289 279 A (IBERICA CYANAMID) 15 November 1995 see claims; examples 6,9-14	1,7,11, 17,19, 24-26
A	WO 96 06619 A (PAUL PREM S ;MENG XIANG JIN (US); HALBUR PATRICK (US); MOROZOV IGO) 7 March 1996 see claims; examples	1-27
A	WO 92 21375 A (STICHTING CENTR DIERGENEESKUND) 10 December 1992 see claims; examples	1,7,11, 19

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
24 September 1998	01/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00251

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	J.J.M. MEULENBERG ET AL.: "Posttranslational Processing and Identification of a Neutralization Domain of the GP4 Protein Encoded by ORF4 of Lelystad Virus" JOURNAL OF VIROLOGY, vol. 71, no. 8, August 1997, pages 6061-6067, XP002045978 see the whole document	1-27

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

In' ational Application No
PCT/NL 98/00251

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		EP 0776209 A	04-06-1997
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		AU 2196592 A	08-01-1993
		CA 2103460 A	07-12-1992
		DE 69201441 D	23-03-1995
		DE 69201441 T	01-06-1995
		DK 587780 T	10-07-1995
		EP 0587780 A	23-03-1994
		ES 2069435 T	01-05-1995
		GR 3015118 T	31-05-1995
		HU 67060 A	30-01-1995
		JP 6508131 T	14-09-1994
		MX 9202724 A	31-12-1992
		US 5620691 A	15-04-1997

(12) UK Patent Application (19) GB (11) 2 289 279 (13) A

(43) Date of A Publication 15.11.1995

(21) Application No 9509392.8

(22) Date of Filing 09.05.1995

(30) Priority Data
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9500815 27.04.1995

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United Kingdom

(51) INT CL[6]
C07K 14/08 , C12N 7/01 15/40 // A61K 39/12 39/295
39/42 , C12N 15/86 , G01N 33/569

(52) UK CL (Edition N)
C3H HB7P HB7T H650 H656 H674
U1S S2419

(56) Documents Cited
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Animal Pharm, No.293, 28Jan. 1994, page 23
Veterinary Microbiology, Vol.33,1992, pages 203-211
Animal Pharm, No. 238,1991,page 20

(58) Field of Search
UK CL (Edition N) C3H HB7P HB7T HB7V
INT CL[6] C07K 14/08
ONLINE: WPI,BIOTECH(DIALOG); CAS ONLINE

(54) Prrsv recombinant proteins

(57) Recombinant proteins of the causative virus of porcine reproductive and respiratory syndrome (PRRS), corresponding to ORFs 2 to 7 of the PRRSV Spanish isolate (PRRS-Olot), have been produced in baculovirus expression system using Sf9 cell cultures as a permissive host. These recombinant proteins are suitable for the formulation of vaccines capable of efficaciously protecting porcine livestock from PRRS and for the preparation of diagnostic kits adequate for detection of anti-PRRSV antibodies as well as of PRRSV in a pig biological sample.

GB 2 289 279 A

FIGURE 1

```
        10          20          30          40          50          60
GAATTGCAGG TAGAGCTAGG TAAACCCCGG CTGCCGCCTG AGCAAGTGCC GTGAATCCGA
        70          80          90         100         110         120
AGTGATGCAA TGGGGTCACT GTGGAGCAAA ATCAGCCAGC TGTTCGTGGA CGCCTTCACT
       130         140         150         160         170         180
GAGTTCCTTG TTAGTGTGGT TGACATTGTC ATTTTCCTTG CCATACTGTT TGGGTTCACC
       190         200         210         220         230         240
GTTGCCGGCT GGTTACTGGT CTTTCTTCTC AGAGTGGTTT GCTCCGCGCT TCTCCGTTCG
       250         260         270         280         290         300
CGCTCTGCCA TTCACTCTCC CGAACTATCG AAGGTCCTAT GAAGGCTTGT TACCCAATTG
       310         320         330         340         350         360
CAGACCGGAT GTCCCACAAT TCGCTGTCAA GCACCCATTG GGTATGTTTT GGCACATGCG
       370         380         390         400         410         420
AGTCTCCCAC CTAATTGATG AAATGGTCTC TCGTCGCATT TACCAGACCA TGGAACATTC
       430         440         450         460         470         480
AGGTCAAGCG GCCTGGAAGC AGGTGGTTAG TGAGGCCACT CTTACAAAGC TGTCAGGACT
       490         500         510         520         530         540
TGATATAGTT ACTCATTTCC AACACCTGGC CGCAGTGGAG GCGGATTCTT GCCGCTTTCT
       550         560         570         580         590         600
CAGCTCACGA CTTGTGATGC TAAAAAATCT TGCCGTTGGC AATGTGAGCC TACAGTACAA
       610         620         630         640         650         660
CACCACGTTA GACCGCGTTG AGCTCATCTT CCCTACGCCG GGTACGAGGC CCAAGTTGAC
       670         680         690         700         710         720
CGATTTCAGA CAATGGCTCA TCAGTGTGCA CGCTTCCATT TTTTCCTCTG TAGCTTCATC
       730         740         750         760         770         780
TGTTACCTTG TTCATAGTGC TTTGGCTTCG AATTCCAATT CTACGCTATG TTTTTGGTTT
```

790 800 810 820 830 840

CCATTGGCCC ACGGCAACAC ATCATTCGAG CTAACCATCA ACTACACCAT ATGTATGCCC

850 860 870 880 890 900

TGCTCTACCA GTCAAGCGGC TCACCAAAGA CTCGAGCCCG GTCGTAACAT GTGGTGCAGA

910 920 930 940 950 960

ATAGGGCACG ACAGGTGTGA GGAACGTGAC CATGATGAGT TGTCAATGTC CATTCCGTCT

970 980 990 1000 1010 1020

GGGTACGATA ACCTCAAACT TGAGGGTTAT TATGCTTGGC TGGCCTTTTT GTCCTTTTCC

1030 1040 1050 1060 1070 1080

TACGCGGCCC AATTCCATCC GGAGTTGTTC GGAATAGGAA ACGTGTCGCG CGTCTTCGTG

1090 1100 1110 1120 1130 1140

GACAAGCAAC ACCAGTTCAT TTGCGCCGAG CATGATGGAC GAAATTCAAC CATATCTACC

1150 1160 1170 1180 1190 1200

GAATATAACA TCTCCGCATT ATATGCGTCG TACTACCATC ACCAAATAGA CGGGGGCAAC

1210 1220 1230 1240 1250 1260

TGGTTCCATT TGGAATGGCT GCGGCCATTC TTTTCCTCCT GGCTGGTGCT CAACATTTCA

1270 1280 1290 1300 1310 1320

TGGTTTCTGA GGCGTTCGCC TGTAAGCCCT GTTTCTCGAC GCATCTATCA GATATTAAGA

1330 1340 1350 1360 1370 1380

CCAACACGAC CGCGGCTGCC GGTTTCATGG TCCTTCAGAA CATCAATTGT CTCCGACCTC

1390 1400 1410 1420 1430 1440

ACGGGGTCTC AACAGCGCAA GAGAACATTT CCTTCGGGAA GCCGTCTCAA TGTCGTGAAG

1450 1460 1470 1480 1490 1500

CCGTCGGTAT TCCCCAGTAC ATTACGATAA CGGCTAATGT GACCGATGAA TCGTATTTGT

1510 1520 1530 1540 1550 1560

ACAACGCGGA CTTGCTGATG CTTTCTGCGT GCCTTTTCTA CGCTTCAGAA ATGAGCGAAA

FIGURE 1 CONT.

1570 1580 1590 1600 1610 1620

AAGGCTTCAA AGTTATCTTT GGGAACGTCT CTGGCGTTGT TTCTGCTTGT GTCAATTTTA

1630 1640 1650 1660 1670 1680

CAGATTATGT GGCCCATGTG ACCCAACATA CCCAGCAGCA TCATCTGGTA ATTGATCACA

1690 1700 1710 1720 1730 1740

TTCGGTTGCT GCATTTCTTG ACACCATCTA CAATGAGGTG GGCTACAACC ATTGCTTGTT

1750 1760 1770 1780 1790 1800

TGTTCGCCAT TCTCTTGGCG ATATGAGATG TTCTCACAAA TTGGGGCGTT TCTTGACTCC

1810 1820 1830 1840 1850 1860

TCACTCTTGC TTCTGGTGGC TTTTTTTGCT GTGTACCGGC TTGTCCTGGT CCTTTGTCGC

1870 1880 1890 1900 1910 1920

TGGCGGCAGC AGCTCGACAT ACCAATACAT ATATAACTTA ACGATATGCG AGCTGAATGG

1930 1940 1950 1960 1970 1980

GACCGACTGG TTGTCCAACC ATTTTGATTG GGCAGTCGAG ACCTTTGTGC TTTACCCGGT

1990 2000 2010 2020 2030 2040

TGCCACTCAT ATCCTCTCAC TGGGTTTTCT CACAACAAGC CATTTTTTTG ACGCGCTCGG

2050 2060 2070 2080 2090 2100

TCTCGGCGCT GTGTCCACTA TAGGATTTGT TGGCGGGCGG TATGTACTCA GCAGCGTGTA

2110 2120 2130 2140 2150 2160

CGGCGCTTGT GCTTTCGCAG CGTTCGTATG TTTTGTCATC CGTGCTGTTA AAAATTGCAT

2170 2180 2190 2200 2210 2220

GGCTTTCCGC TATGCCCACA CCCGGTTTAC CAACTTCATT GTGGACGACC GGGGGAGAAT

2230 2240 2250 2260 2270 2280

CCATCGGTGG AAGTCTCCAA TAGTGGTAGA GAAATTGGGC AAAGCTGAAG TCGGTGGCGA

2290 2300 2310 2320 2330 2340

CCTTGTCACC ATCAAACATG TCGTCCTCGA AGGGGTTAAA GCTCAACCCT TGACGAGGAC

FIGURE 1 CONT.

2350 2360 2370 2380 2390 2400

TTCGGCTGAG CAATGGGAAG CCTAGACGAT TTTTGCAATG ATTCTACCGC CGCACAAAAG

2410 2420 2430 2440 2450 2460

CTTGTGCTAG CCTTTAGCAT TACATATACA CCTATAATGA TATACGCCCT TAAGGTGTCA

2470 2480 2490 2500 2510 2520

CGCGGCCGAC TCCTGGGGCT GTTGCACATC CTAATATTCC TGAATTGTTC TTTCACATTC

2530 2540 2550 2560 2570 2580

GGATACATGA CATATGTGCG TTTTCAATCC ACCAACCGTG TCGCACTTAC TCTGGGGGCT

2590 2600 2610 2620 2630 2640

GTTGTCGCCC TTCTGTGGGG TGTTTACAGC TTCACAGAGT CATGGAAGTT TGTTACTTCC

2650 2660 2670 2680 2690 2700

AGATGCAGAT TGTGTTGCCT AGGCCGGCGA TACATTCTGG CCCCTGCCCA TCACGTAGAA

2710 2720 2730 2740 2750 2760

AGTGCTGCAG GTCTCCATTC AATCCCAGCG TCTGGTAACC GAGCATACGC TGTGAGAAAG

2770 2780 2790 2800 2810 2820

CCCGGACTAA CATCAGTGAA CGGCACTCTA GTTCCAGGAC TTCGGAGCCT CGTGCTGGGC

2830 2840 2850 2860 2870 2880

GGCAAACGAG CTGTTAAACG AGGAGTGGTT AACCTCGTCA AGTATGGCCG GTAAAAACCA

2890 2900 2910 2920 2930 2940

GAGCCAGAAG AAAAAGAAAA GTGCAGCTCC GATGGGGAAT GGCCAGCCAG TCAATCAACT

2950 2960 2970 2980 2990 3000

GTGCCAGTTG CTGGGTGCAA TGATAAAGTC CCAGCGCCAG CAACCTAGGG GAGGACAGGC

3010 3020 3030 3040 3050 3060

CAAAAAGAAA AAGCCTGAGA AGCCACATTT TCCCTTAGCT GCTGAAGATG ACATCCGGCA

3070 3080 3090 3100 3110 3120

CCACCTCACC CAGACCGAAC GTTCCCTCTG CTTGCAATCG ATCCAGACGG CTTTTAATCA

FIGURE 1 CONT.

```
      3130        3140        3150        3160        3170        3180
AGGCGCAGGA ACTGCGTCGC TTTCATCCAG CGGGAAGGTC AGTTTTCAGG TTGAGTTCAT
      3190        3200        3210        3220        3230        3240
GCTGCCGGTT GCTCATACGG TGCGCCTGAT TCGCGTGACT TCTACATCCG CCAGTCAGGG
      3250        3260        3270        3280        3290        3300
TGCAAGCTAA TTTGACAGTC AGGTGAATGG CCGCGATTGA CGTGTGGCCT CTAAGTCACC
      3310        3320        3330        3340        3350        3360
TATTCAATTA GGGCGATCAC ATGGGGGTCA AACTTAATCA GGCAGGAACC ATGTGACCGA
      3370        3380
AATTAAAAAA AAAAAAAAAA AAA
```

FIGURE 1 CONT.

ORF2

```
Met Gln Trp Gly His Cys Gly Ala Lys Ser Ala Ser Cys Ser Trp Thr
 1               5                  10                  15

Pro Ser Leu Ser Ser Leu Leu Val Trp Leu Thr Leu Ser Phe Ser Leu
            20                  25                  30

Pro Tyr Cys Leu Gly Ser Pro Leu Pro Ala Gly Tyr Trp Ser Phe Phe
        35                  40                  45

Ser Glu Trp Phe Ala Pro Arg Phe Ser Val Arg Ala Leu Pro Phe Thr
    50                  55                  60

Leu Pro Asn Tyr Arg Arg Ser Tyr Glu Gly Leu Leu Pro Asn Cys Arg
65                  70                  75                  80

Pro Asp Val Pro Gln Phe Ala Val Lys His Pro Leu Gly Met Phe Trp
                85                  90                  95

His Met Arg Val Ser His Leu Ile Asp Glu Met Val Ser Arg Arg Ile
            100                 105                 110

Tyr Gln Thr Met Glu His Ser Gly Gln Ala Ala Trp Lys Gln Val Val
        115                 120                 125

Ser Glu Ala Thr Leu Thr Lys Leu Ser Gly Leu Asp Ile Val Thr His
    130                 135                 140

Phe Gln His Leu Ala Ala Val Glu Ala Asp Ser Cys Arg Phe Leu Ser
145                 150                 155                 160

Ser Arg Leu Val Met Leu Lys Asn Leu Ala Val Gly Asn Val Ser Leu
                165                 170                 175

Gln Tyr Asn Thr Thr Leu Asp Arg Val Glu Leu Ile Phe Pro Thr Pro
            180                 185                 190

Gly Thr Arg Pro Lys Leu Thr Asp Phe Arg Gln Trp Leu Ile Ser Val
        195                 200                 205

His Ala Ser Ile Phe Ser Ser Val Ala Ser Ser Val Thr Leu Phe Ile
    210                 215                 220

Val Leu Trp Leu Arg Ile Pro Ile Leu Arg Tyr Val Phe Gly Phe His
225                 230                 235                 240

Trp Pro Thr Ala Thr His His Ser Ser
                245
```

FIGURE 2A

ORF3

```
Met Ala His Gln Cys Ala Arg Phe His Phe Phe Leu Cys Ser Phe Ile
 1               5                  10                  15

Cys Tyr Leu Val His Ser Ala Leu Ala Ser Asn Ser Asn Ser Thr Leu
            20                  25                  30

Cys Phe Trp Phe Pro Leu Ala His Gly Asn Thr Ser Phe Glu Leu Thr
        35                  40                  45

Ile Asn Tyr Thr Ile Cys Met Pro Cys Ser Thr Ser Gln Ala Ala His
    50                  55                  60

Gln Arg Leu Glu Pro Gly Arg Asn Met Trp Cys Arg Ile Gly His Asp
65                  70                  75                  80

Arg Cys Glu Glu Arg Asp His Asp Glu Leu Ser Met Ser Ile Pro Ser
                85                  90                  95

Gly Tyr Asp Asn Leu Lys Leu Glu Gly Tyr Tyr Ala Trp Leu Ala Phe
            100                 105                 110

Leu Ser Phe Ser Tyr Ala Ala Gln Phe His Phe Glu Leu Phe Gly Ile
        115                 120                 125

Gly Asn Val Ser Arg Val Phe Val Asp Lys Gln His Gln Phe Ile Cys
    130                 135                 140

Ala Glu His Asp Gly Arg Asn Ser Thr Ile Ser Thr Glu Tyr Asn Ile
145                 150                 155                 160

Ser Ala Leu Tyr Ala Ser Tyr Tyr His His Gln Ile Asp Gly Gly Asn
                165                 170                 175

Trp Phe His Leu Glu Trp Leu Arg Pro Phe Phe Ser Ser Trp Leu Val
            180                 185                 190

Leu Asn Ile Ser Trp Phe Leu Arg Arg Ser Pro Val Ser Pro Val Ser
        195                 200                 205

Arg Arg Ile Tyr Gln Ile Leu Arg Pro Thr Arg Pro Arg Leu Pro Val
    210                 215                 220

Ser Trp Ser Phe Arg Thr Ser Ile Val Ser Asp Leu Thr Gly Ser Gln
225                 230                 235                 240

Gln Arg Lys Arg Thr Phe Pro Ser Gly Ser Arg Leu Asn Val Val Lys
                245                 250                 225

Pro Ser Val Phe Pro Ser Thr Leu Arg
            260                 265
```

FIGURE 2B

ORF4

```
Met Ala Ala Ala Ile Leu Phe Leu Leu Ala Gly Ala Gln His Phe Met
  1               5                  10                  15

Val Ser Glu Ala Phe Ala Cys Lys Pro Cys Phe Ser Thr His Leu Ser
            20                  25                  30

Asp Ile Lys Thr Asn Thr Thr Ala Ala Ala Gly Phe Met Val Leu Gln
        35                  40                  45

Asn Ile Asn Cys Leu Arg Pro His Gly Val Ser Thr Ala Gln Glu Asn
    50                  55                  60

Ile Ser Phe Gly Lys Pro Ser Gln Cys Arg Glu Ala Val Gly Ile Pro
65                  70                  75                  80

Gln Tyr Ile Thr Ile Thr Ala Asn Val Thr Asp Glu Ser Tyr Leu Tyr
                85                  90                  95

Asn Ala Asp Leu Leu Met Leu Ser Ala Cys Leu Phe Tyr Ala Ser Glu
            100                 105                 110

Met Ser Glu Lys Gly Phe Lys Val Ile Phe Gly Asn Val Ser Gly Val
        115                 120                 125

Val Ser Ala Cys Val Asn Phe Thr Asp Tyr Val Ala His Val Thr Gln
    130                 135                 140

His Thr Gln Gln His His Leu Val Ile Asp His Ile Arg Leu Leu His
145                 150                 155                 160

Phe Leu Thr Pro Ser Thr Met Arg Trp Ala Thr Thr Ile Ala Cys Leu
                165                 170                 175

Phe Ala Ile Leu Leu Ala Ile
            180
```

FIGURE 2C

ORF5

```
Met Arg Cys Ser His Lys Leu Gly Arg Phe Leu Thr Pro His Ser Cys
 1               5                  10                  15

Phe Trp Trp Leu Phe Leu Leu Cys Thr Gly Leu Ser Trp Ser Phe Val
            20                  25                  30

Ala Gly Gly Ser Ser Ser Thr Tyr Gln Tyr Ile Tyr Asn Leu Thr Ile
        35                  40                  45

Cys Glu Leu Asn Gly Thr Asp Trp Leu Ser Asn His Phe Asp Trp Ala
    50                  55                  60

Val Glu Thr Phe Val Leu Tyr Pro Val Ala Thr His Ile Leu Ser Leu
65                  70                  75                  80

Gly Phe Leu Thr Thr Ser His Phe Phe Asp Ala Leu Gly Leu Gly Ala
                85                  90                  95

Val Ser Thr Ile Gly Phe Val Gly Gly Arg Tyr Val Leu Ser Ser Val
            100                 105                 110

Tyr Gly Ala Cys Ala Phe Ala Ala Phe Val Cys Phe Val Ile Arg Ala
        115                 120                 125

Val Lys Asn Cys Met Ala Cys Arg Tyr Ala His Thr Arg Phe Thr Asn
    130                 135                 140

Phe Ile Val Asp Asp Arg Gly Arg Ile His Arg Trp Lys Ser Pro Ile
145                 150                 155                 160

Val Val Glu Lys Leu Gly Lys Ala Glu Val Gly Gly Asp Leu Val Thr
                165                 170                 175

Ile Lys His Val Val Leu Glu Gly Val Lys Ala Gln Pro Leu Thr Arg
            180                 185                 190

Thr Ser Ala Glu Gln Trp Glu Ala
        195                 200
```

FIGURE 2D

ORF6

Met Gly Ser Leu Asp Asp Phe Cys Asn Asp Ser Thr Ala Ala Gln Lys
1 5 10 15

Leu Val Leu Ala Phe Ser Ile Thr Tyr Thr Pro Ile Met Ile Tyr Ala
20 25 30

Leu Lys Val Ser Arg Gly Arg Leu Leu Gly Leu Leu His Ile Leu Ile
35 40 45

Phe Leu Asn Cys Ser Phe Thr Phe Gly Tyr Met Thr Tyr Val Arg Phe
50 55 60

Gln Ser Thr Asn Arg Val Ala Leu Thr Leu Gly Ala Val Val Ala Leu
65 70 75 80

Leu Trp Gly Val Tyr Ser Phe Thr Glu Ser Trp Lys Phe Val Thr Ser
85 90 95

Arg Cys Arg Leu Cys Cys Leu Gly Arg Arg Tyr Ile Leu Ala Pro Ala
100 105 110

His His Val Glu Ser Ala Ala Gly Leu His Ser Ile Pro Ala Ser Gly
115 120 125

Asn Arg Ala Tyr Ala Val Arg Lys Pro Gly Leu Thr Ser Val Asn Gly
130 135 140

Thr Leu Val Pro Gly Leu Arg Ser Leu Val Leu Gly Gly Lys Arg Ala
145 150 155 160

Val Lys Arg Gly Val Val Asn Leu Val Lys Tyr Gly Arg
165 170

FIGURE 2E

ORF7

```
Met Ala Gly Lys Asn Gln Ser Gln Lys Lys Lys Lys Ser Ala Ala Pro
 1               5                  10                  15

Met Gly Asn Gly Gln Pro Val Asn Gln Leu Cys Gln Leu Leu Gly Ala
            20                  25                  30

Met Ile Lys Ser Gln Arg Gln Gln Pro Arg Gly Gly Gln Ala Lys Lys
        35                  40                  45

Lys Lys Pro Glu Lys Pro His Phe Pro Leu Ala Ala Glu Asp Asp Ile
    50                  55                  60

Arg His His Leu Thr Gln Thr Glu Arg Ser Leu Cys Leu Gln Ser Ile
65                  70                  75                  80

Gln Thr Ala Phe Asn Gln Gly Ala Gly Thr Ala Ser Leu Ser Ser Ser
                85                  90                  95

Gly Lys Val Ser Phe Gln Val Glu Phe Met Leu Pro Val Ala His Thr
            100                 105                 110

Val Arg Leu Ile Arg Val Thr Ser Thr Ser Ala Ser Gln Gly Ala Ser
        115                 120                 125
```

FIGURE 2F

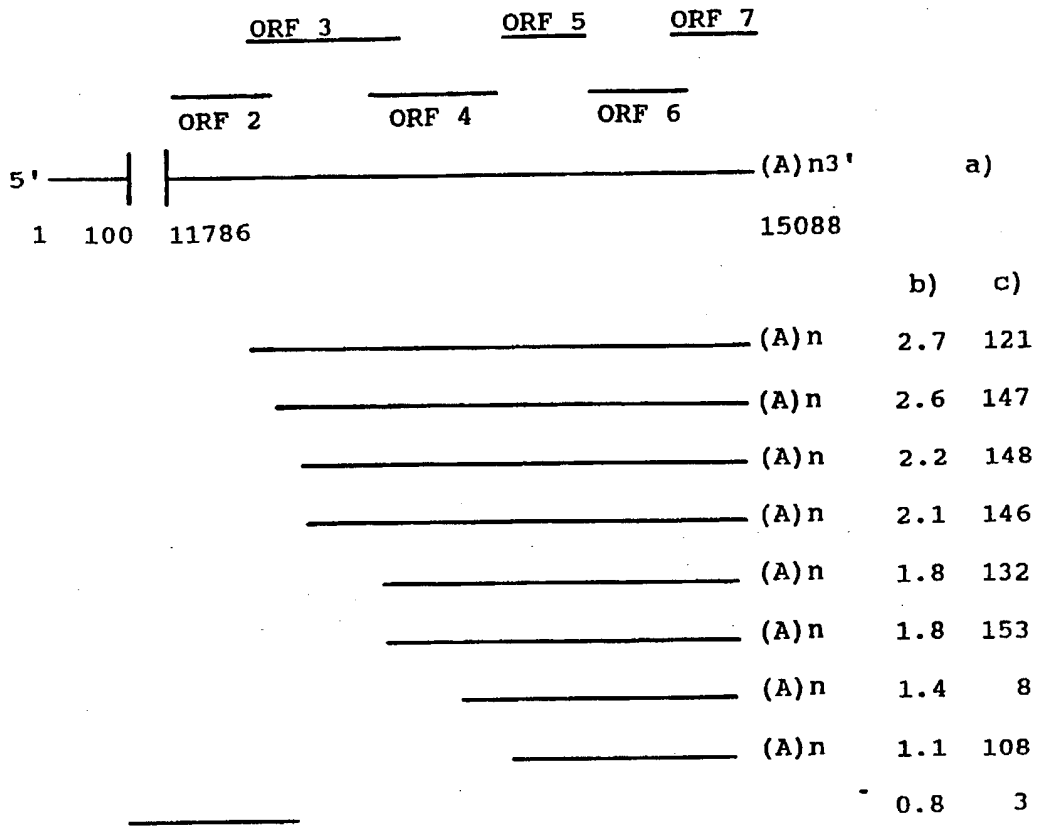

ORF 3
ORF 5
ORF 7
ORF 2
ORF 4
ORF 6
5'
(A)n3'
a)
1
100
11786
15088
b)
c)
(A)n 2.7 121
(A)n 2.6 147
(A)n 2.2 148
(A)n 2.1 146
(A)n 1.8 132
(A)n 1.8 153
(A)n 1.4 8
(A)n 1.1 108
0.8 3

FIGURE 3

pPRRS-3

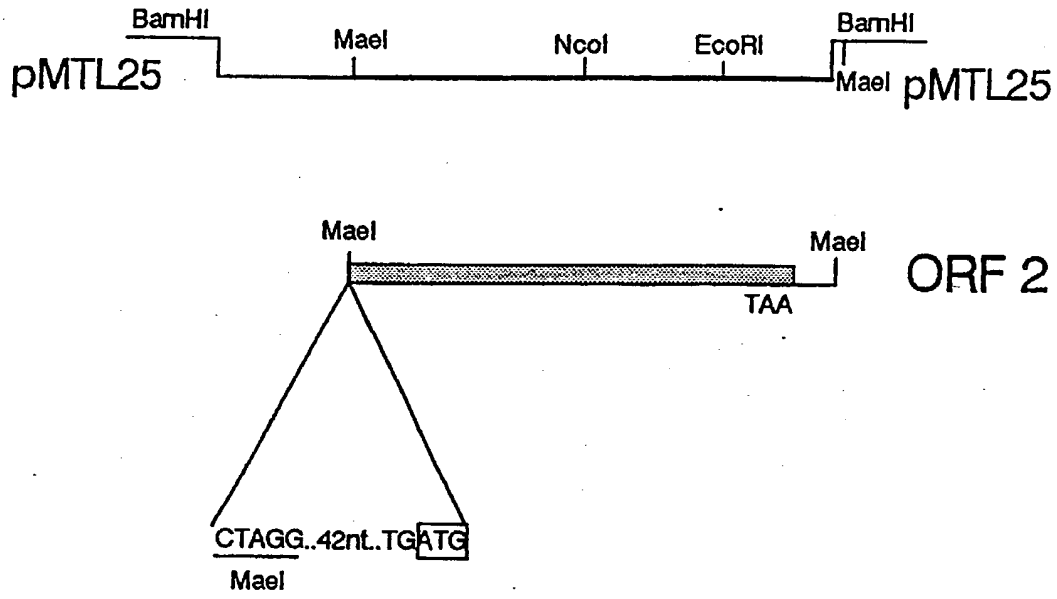


FIGURE 4

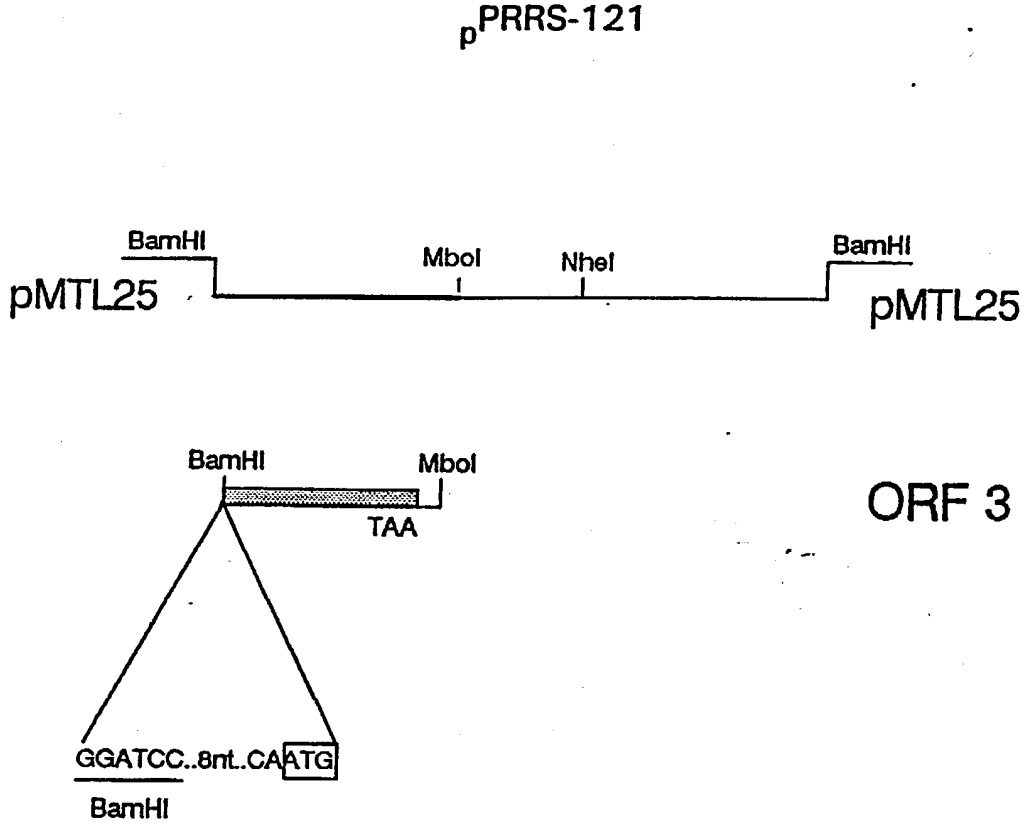

pPRRS-121
BamHI
MboI
NheI
BamHI
pMTL25
pMTL25
BamHI
MboI
TAA
ORF 3
GGATCC..8nt..CAATG
BamHI

FIGURE 5

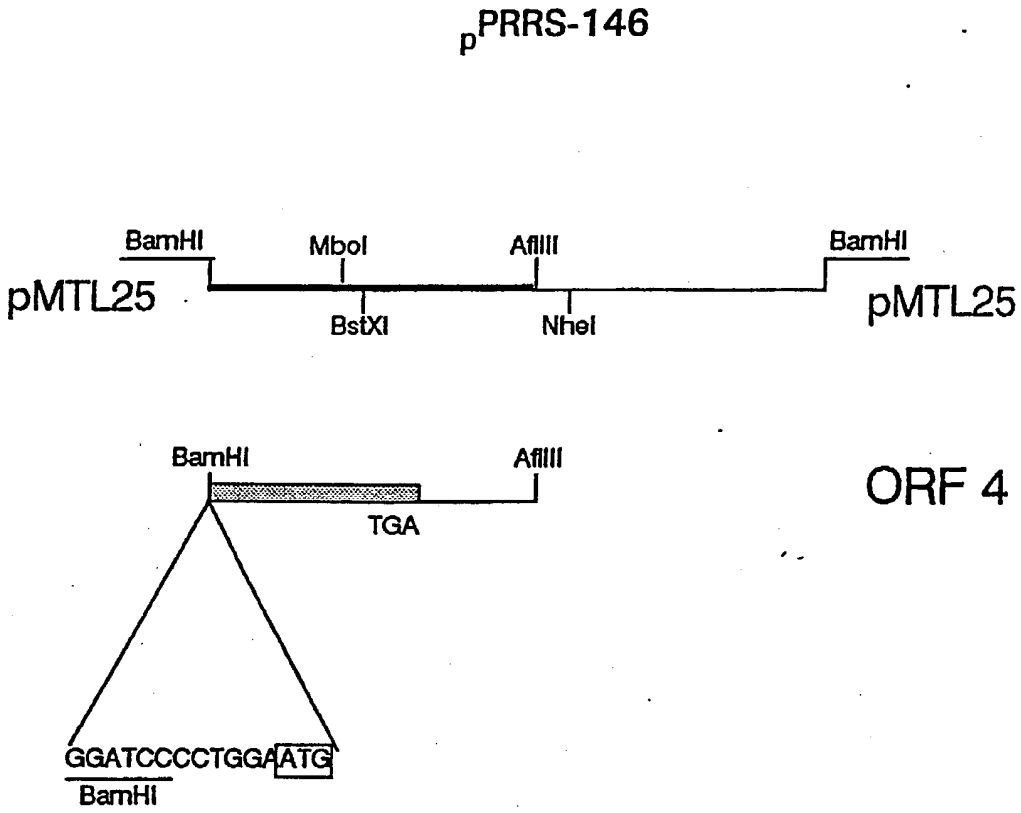

pPRRS-146
BamHI
MboI
AflIII
BamHI
pMTL25
BstXI
NheI
pMTL25
BamHI
AflIII
ORF 4
TGA
GGATCCCCTGGAATG
BamHI

FIGURE 6

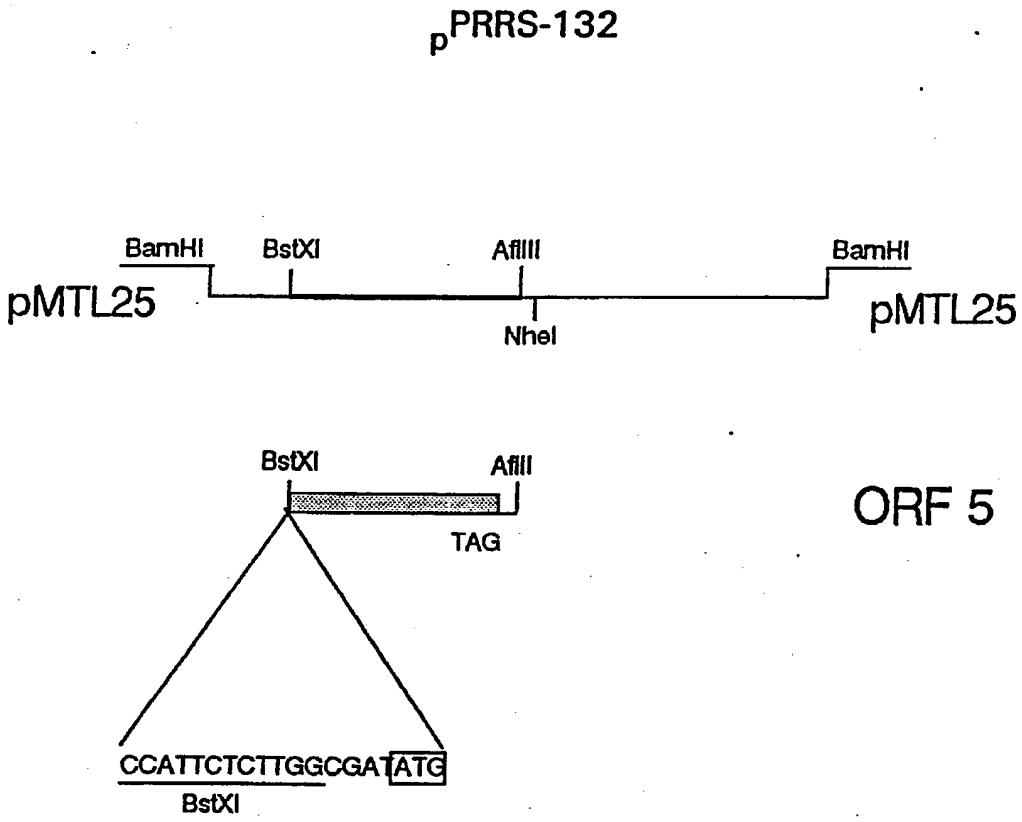

pPRRS-132
BamHI
BstXI
AflIII
BamHI
pMTL25
NheI
pMTL25
BstXI
AflII
TAG
ORF 5
CCATTCTCTTGGCGATATG
BstXI

FIGURE 7

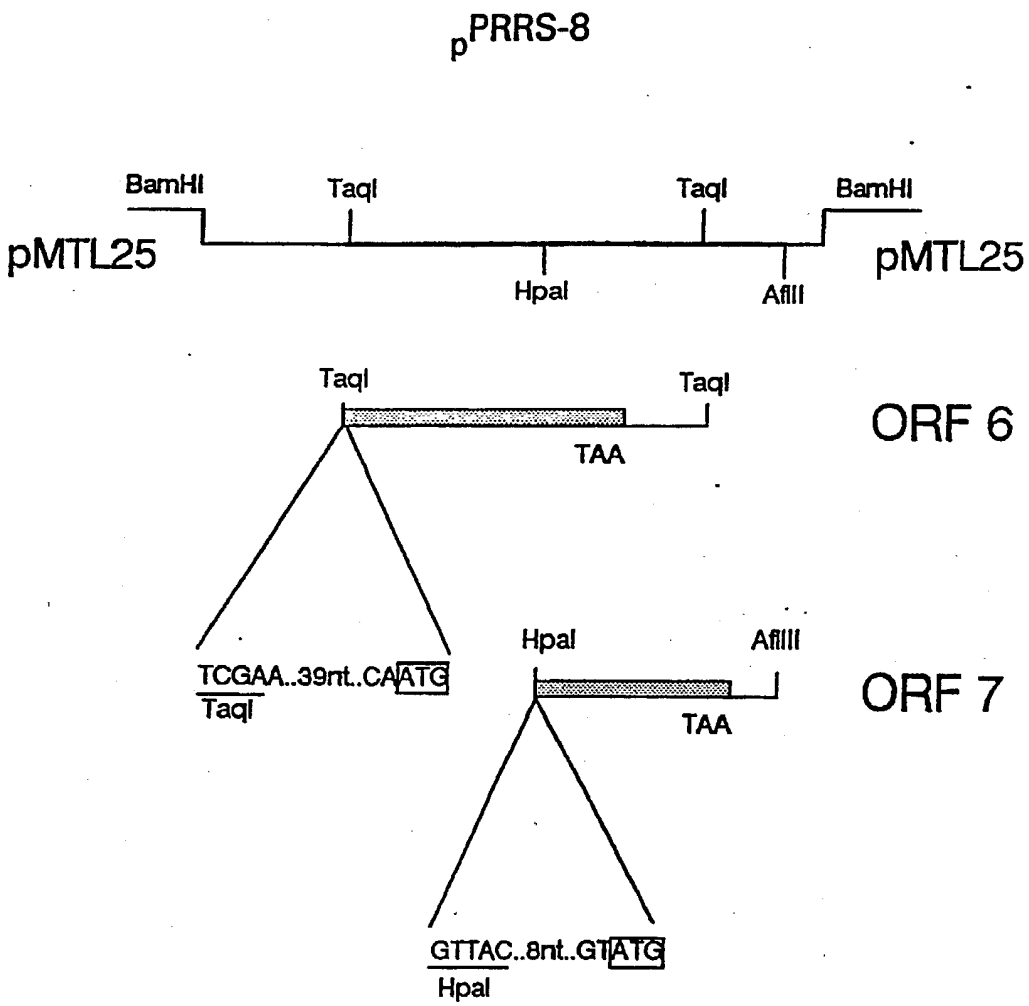
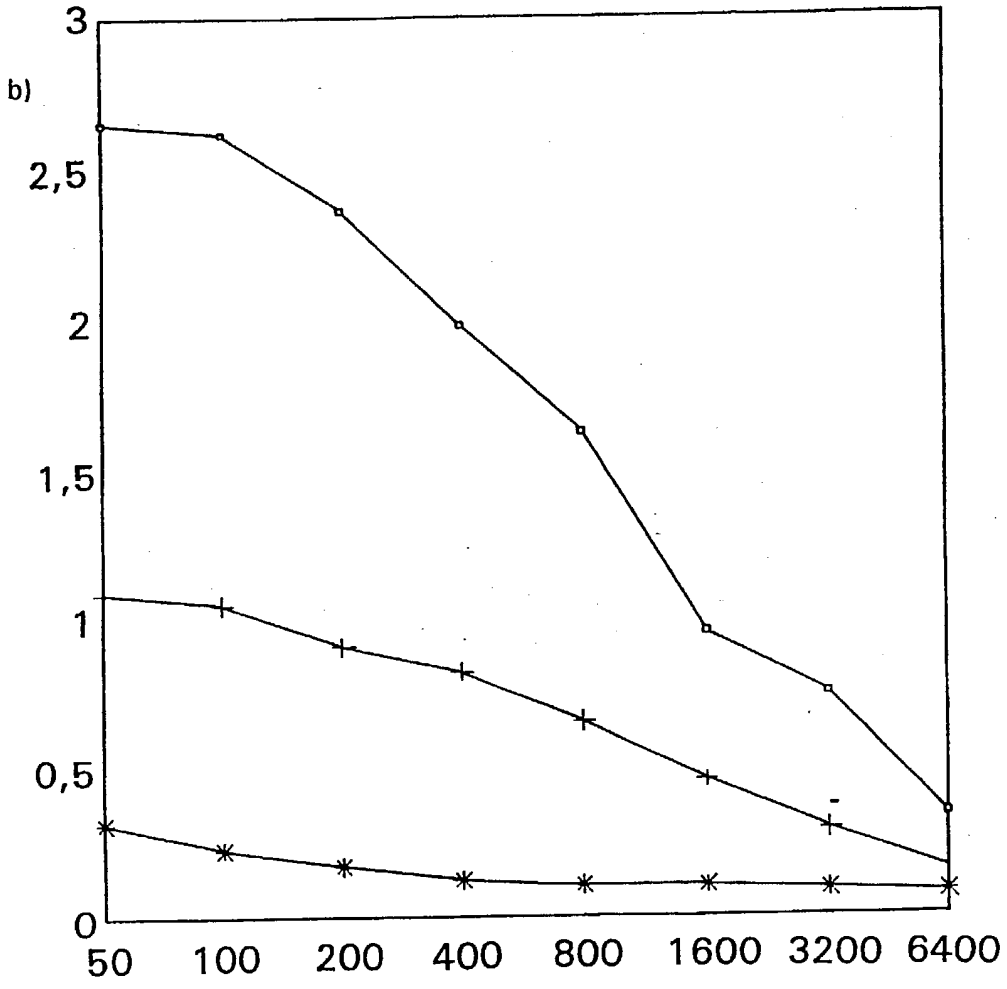

pPRRS-8
BamHI
TaqI
TaqI
BamHI
pMTL25
HpaI
AflII
pMTL25
TaqI
TaqI
TAA
ORF 6
TCGAA..39nt..CAATG
TaqI
HpaI
AflII
TAA
ORF 7
GTTAC..8nt..GTATG
HpaI

FIGURE 8

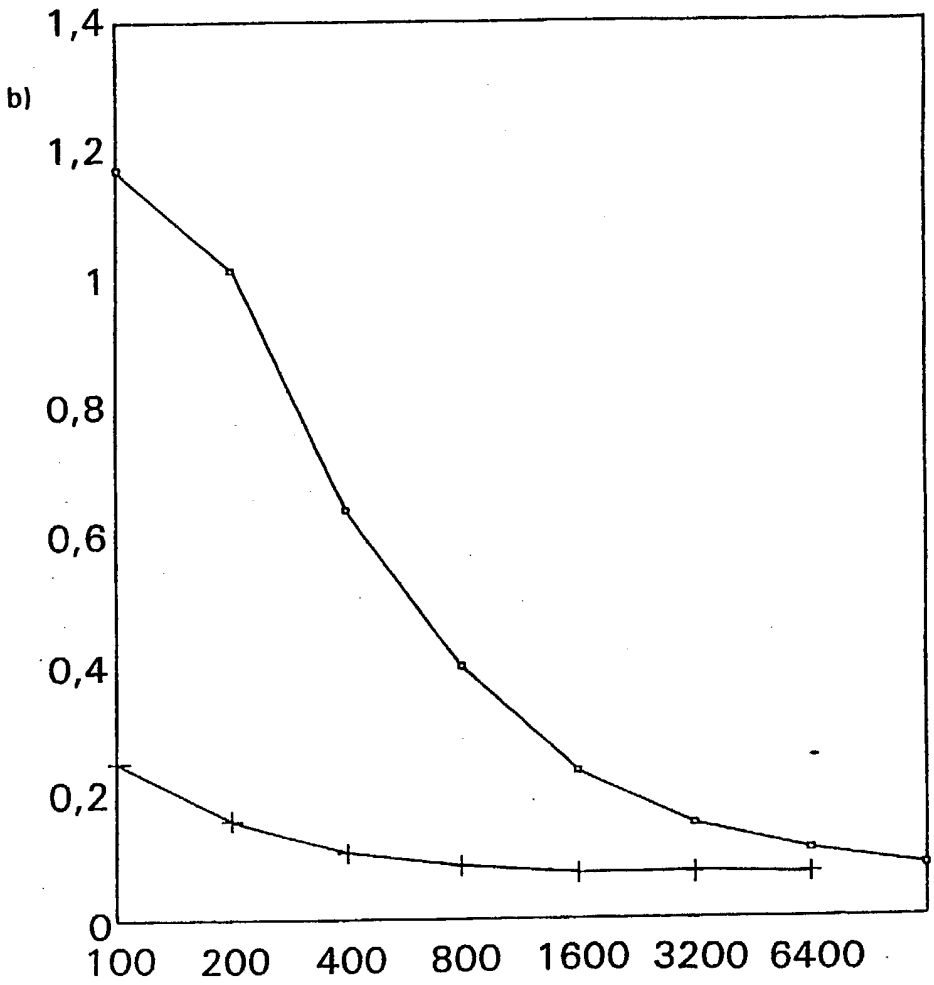
a)



c) d)

FIGURE 9

a)

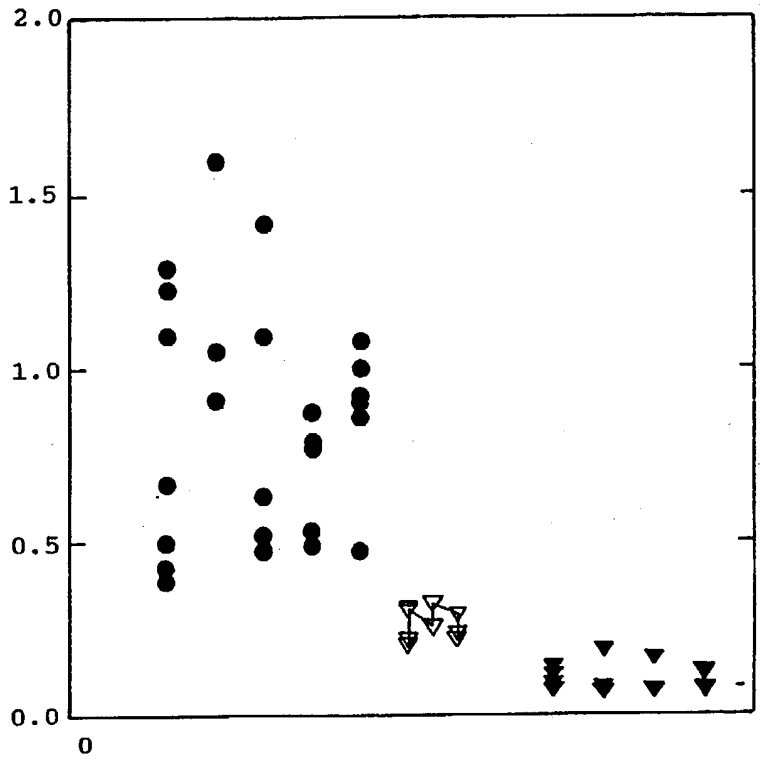


c) d)

FIGURE 10

a)

b)



c)

FIGURE 11

2289279

RECOMBINANT PRRSV PROTEINS, DIAGNOSTIC KITS AND VACCINES CONTAINING SUCH RECOMBINANT PRRSV PROTEINS

SCOPE OF THE INVENTION

This invention relates to viral recombinant proteins of the causative agent of porcine reproductive and respiratory syndrome (PRRS) produced in an expression system of recombinant baculoviruses multiplied in permissive host cell culture. The invention also relates to diagnostic kits and vaccines which comprise, at least, one of the said recombinant proteins.

HISTORY OF THE INVENTION

In Spain, the first cases of respiratory alterations in piglets were detected in a 300-piglet batch imported from Germany, in mid-January 1991 (Plana et al., Med. Vet., Vol. 8, No. 11, 1991). Shortly afterwards, in two breeding herds on two farms situated near the herd where the initial problem had appeared, a disease was detected characterized by an abnormally high number of abortions during the last phase of gestation, as well as 70% mortality in piglets.

The cause of these epizootic outbreaks was not known, but their symptomatology was similar to the clinical signs that had been described for a swine disease first detected in Europe in Germany (1991), and to the disease denominated Mystery Swine Disease detected in the United States and Canada in 1987 (Hill, Proceedings of the Mystery Swine Disease Committee Meeting, October 6, 1990, Denver, USA). This disease affects pregnant sows, provoking in them anorexia, abortions, stillbirths, mummified fetuses, weak piglets that die in a few hours of life, and post-farrowing respiratory problems, among others. At present,

the disease is known as "Porcine Reproductive and Respiratory Syndrome" (PRRS), although it was previously referred to as "Blue-eared Pig Disease", "Mysterious Reproductive Syndrome" (MRS), "Swine Infertility and Respiratory Syndrome" (SIARS) and "Porcine Epidemic Abortion and Respiratory Syndrome" (PEARS).

At present, it is known that the causative agent of this disease is a virus donominated as PRRS virus (PRRSV). This virus was isolated for the first time in the Netherlands by a group of researchers of the CDI/Lelystad, who denominated it as Lelystad virus (LV) (Wesvoort, G et al., Vet. Quarterly, Vol 3, 121-130, 1991). Some months later, another isolate was obtained in Spain by Laboratorios Sobrino/Cyanamid (Plana et al., Vet. Microbiol., 33:203.211, 1992), which will be identified in this description as PRRS-Olot. From that time, new isolates of this virus have been described (EP Requests No. 0 529 584 A2, PCT Requests Nos. WO 93/06211 and WO 93/07898).

The structural characteristics of the PRRS virus have been described in two recent publications:

a) Meulenberg, J.J.M., et al., "Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV". Virology, 192: 62-72, (1993); and

b) Cozelmann, K-K., et al., "Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group". Virology, 193: 329-339, (1993).

The PRRS virus has a size of 50-60 nm, with an envelope of of approximately 30-35 nm contained in the nucelocapsid, and a single RNA molecule as genomic material. Based on these morphological data, PRRSV was initially classified as a Togavirus, although based on its genomic structure

and transcription and translation mechanisms it was closer to the Coronaviridae family. Recently, and based on differences and/or similarities in comparison with the previous groups, its classification was proposed within a new family denominated Arteriviridae (Cavanagh D., et al., Arch. Virology, 1994). Together with PRRSV, in this group are included the equine arteritis viruses (EAV), lactic dehydrogenase virus (LDV) and simian hemorrhagic fever virus (SHFV).

Recently, the entire Lelystad virus (LV) genome (Meulenberg et al., quoted above), a genomic segment of the Tübingen (Germany) PRRS virus isolate (TV)(Cozelmann et al., quoted above), and a segment of the PRRS-Olot virus (Spanish Patent claim no. ES P9301973) were cloned and sequenced. Based on all the results obtained it can be stated that the PRRSV genome is made up of a single strand RNA molecule which contains at 3' end a poly-A tail. The length of the genome is of approximately 15000 base pairs (bp), and in its structure it contains seven open reading frames (ORFs) coding for the viral proteins. The ORFs have been denominated as ORF1 to ORF7 and they show small overlapping segments between them. It has been propounded that synthesis of the viral proteins is produced from a group of different length subgenomic transcripts (mRNA), but of similar 3' polyadenilated end, and 5' leader sequence originating from the non-coding 5' end sequence. This form of viral protein expression has been denominated as nested mRNAs and has been previously described for coraniviruses (Spaan, W.J.M., Cavanagh, D., and Horzineck, M.C., J. Gen. Virol., 69:2939-2952, 1988). Based on the Lelystad (LV) and Tübingen (TV) PRRSV viral isolate nucleotide sequence, and by homology with what has been observed with other arteriviruses, it has been propounded that in the viral genome, ORF1 (a and b) code

for viral polymerase and replicase. ORFs 2 to 6 would code for the viral envelope proteins, and ORF7 would code for the neuclocapsid protein. Viral replicase and polymerase are large-sized proteins, 260 and 163 kDa respectively, and both of them contain three possible glycosilation sites. Envelope proteins (ORFs 2 to 6) located at 3' end are small, between 30 and 19 kDa. All of them contain more than two possible glycosilation sites, especially ORF3 which contains 7 sites. All of these proteins contain hydrophobic sequences at the amino (N-) and carboxy (C-) terminal ends that might work as leader sequence and membrane anchor. Generally, they are hydrophobic proteins, in accordance with their location associated to a membrane. ORF6 should be pointed out, with 3 hydrophobic segments located within the 90 amino acid residues at the N-terminal end. On the other hand, the protein coded by ORF7, possibly corresponding to the viral nucleocapsid, is extremely basic with arginine, lysine and histidine residues at the N-terminal end. The amino acid sequences of LV and TV viral polymerase, structural proteins and nucleocapsid show an identity of between 29% and 67% in comparison with LDV virus, and between 20% and 36% in comparison with EAV virus. This suggests that the evolution of the PRRS virus is closer to LDV than to EAV.

The disease caused by PRRSV is responsible for severe losses to the pig industry. For this reason, vaccines capable of preventing the infection caused by PRRSV have been developed.

In general, the vaccines against known PRRSV, described in patent claims WO 92/21375, WO 93/06211, WO 93/07898 and ES P9301973 are vaccines obtained from viruses grown on macrophages and subsequently inactivated. Patent application ES P9301973 provides a vaccine capable of

avoiding porcine reproductive and respiratory syndrome (PRRS). The vaccine has been demonstrated to be efficacious in avoiding reproductive alterations in sows, such as the farrowing of stillborn, mummified or living but weak piglets, repetition of estrus and similar problems produced by the virus causative of PRRS. Likewise, it has been verified that the vaccine induces cellular immunity in the vaccinated animals. The said vaccine contains a suitable quantity of PRRS viral antigen, Spanish strain (PRRS-Olot), inactivated, together with an adjuvant and preservative.

The present invention provides a second generation vaccine in which recombinant DNA technology has been employed with the objective of obtaining new vaccines capable of efficaciously protecting against the infection caused by PRRSV. The vaccines of this invention contain, at least, one recombinant PRRSV protein. On the other hand, the present invention provides new PRRSV diagnostic systems or kits that involve the use of enzymatic immunoassay techniques (ELISA) that use recombinant PRRSV proteins. These recombinant vaccines do not require manipulation of the complete virus, but rather of only part of it, eliminating the risk of an accident that would free virus, representing a considerable advantage over the present inactivated PRRSV vaccines. These new recombinant vaccines do not require manipulation of the complete virus, but rather of only part of it, eliminating the risk of an accident that would free virus, which represents a considerable advantage over the present inactivated PRRSV vaccines.

The production of recombinant proteins by means of Genetic Engineering is a fact that has been described previously. Numerous expression and production systems of recombinant proteins are known. One of the most effective systems for

large-scale production of recombinant proteins is based on the replication of recombinant baculoviruses derived from the *Autographa californica* nuclear polyhedrosis virus (AcNPV), in insect cells in culture. The description of the baculovirus expression technique is described in the following articles:

a) LucKow, V.A. & Summers, M.D., "Trends in the development of baculovirus expression vectors". Bio/Technology, 6:47-55, (1988) ; and

b) Bishop, D.H.L., "Baculovirus expression vectors". Seminars in VIROLOGY, 3:253-264 (1992).

This invention provides recombinant PRRSV proteins, in particular of the PRRS-Olot isolate, produced in an expression system of baculoviruses multiplied on permissive host cell culture. The recombinant baculoviruses capable of producing such recombinant proteins, as well as the transfer vectors used, constitute additional objectives of the invention. The procedures for the obtainment of such recombinant baculoviruses and proteins is also an objective of this invention.

The invention provides also new vaccines for the vaccination of pigs for their protection against the infection caused by PRRSV, comprising, at least, one recombinant protein of those provided by this invention and an adequate carrier or adjuvant.

The invention provides also a diagnostic kit to detect the presence of antibodies that specifically recognize PRRSV in a biological sample from pigs (e.g.: blood, serum, sputum, saliva or milk). The kit comprises at least one recombinant protein of those provided by this invention and adequate detection methods.

The invention provides also a diagnostic kit for the detection of the presence of antigen (PPRSV) in a biological sample from pigs (e.g.: blood, serum, sputum,

saliva, milk or tissue). The kit comprises at least one antibody which specifically recognizes PRRSV obtained by immunizing animals with, at least, one recombinant protein of those provided by this invention and adequate detection means.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the consecutive sequence of the 3383 bp cloned from the PRRS-Olot isolate.

Figure 2 shows the amino acid sequence corresponding to the proteins coded by ORF2 (Figure 2A), ORF3 (Figure 2B), ORF4 (Figure 2C), ORF5 (Figure 2D), ORF6 (Figure 2E) and ORF7 (Figure 2F).

Figure 3 shows the different extension of clones pPRRS-8, pPRRS-108, pPRRS-121, pPRRS-132, pPPRS-146, pPPRS-147, pPPRS-148, pPRRS-153 and pPRRS-3, in comparison with LV, as well as the ORFs contained in each one of them. In this figure, reference is made to the PRRSV genome (a), size in Kb (b) and number of the clone (c).

Figure 4 shows pPRRS-3 clone containing the gene of the protein coded by ORF2.

Figure 5 shows pPRRS-121 clone containing the gene of the protein coded by ORF3.

Figure 6 shows pPRRS-146 clone containing the gene of the protein coded by ORF4.

Figure 7 shows pPRRS-132 clone containing the gene of the protein coded by ORF5.

Figure 8 shows pPRRS-8 clone containing the genes of the proteins coded by ORF6 and ORF7.

Figure 9 shows the results from antigen titration by ELISA (absorbance monitored at 405 nm). Figure 9 shows the

results of antigen titration by ELISA. In the figure reference is made to antigen titration (a), absorbance values read at 405 nm (b), and antigen dilutions [in units of 1/] (c).

Figure 10 shows the results from the titration, by ELISA, of a PRRS field serum obtained in an infected animal. The figure makes reference to the titration of the serum (a), absorbance values read at 405 nm (b), and serum dilutions [in units of 1/] (c).

Figure 11 shows the results obtained from a sampling experiment with several dozen field sera. The figure makes reference to the titration of the sera (a), absorbance values read at 405 nm (b), and the sera (c)

DESCRIPTION OF THE INVENTION

Our Laborotry has made a search for the PRRS causative agent in recent years. The main consequence of this has been the isolation of the virus denominated PRRS-CY-JPD-P5-6-91. It was deposited at the ECACC (with accession number V93070108) and a vaccine was developed against PRRSV containing the inactivated virus (Patent Application ES P9301973).

Since then, our research efforts have addressed the isolation and cloning of the PRRSV (PRRS-CY-JPD-P5-6-91) genome, denominated as PRRS-Olot in this description, in order to enable the development of new recombinant vaccines effective against the infection caused by PRRSV. To that end, a genome segment of the said PRRS-Olot genome has been cloned. The cloned fragment corresponds to the 3' viral genome, and represents a consecutive sequence of 3338 bp. This segment contains the six open reading frames corresponding to ORFs 2 to 7 described for LV and TV. They code for the structural proteins of the virus (nucleocapsid and envelope) possibly involved in viral

antigenicity and immunogenicity. The proteins coded by PRRS-Olot ORFs 2 to 7 are similar to the corresponding LV and TV proteins. Their characteristics are summarized in Table 1, where are indicated, in relation with each ORF, the relative positions of the nucleotides, the number of base pairs (bp), the number of amino acids (Aac), the molecular weight of each protein (in KDa) and the glycosilation sites.

Table 1

Characteristics of the PRRS-Olot virus ORFs

ORF	Nucleotides (site)	bp	Aac (No.)	Protein (KDa)	Glyco-silation
2	65-811	747	249	28.4	2
3	673-1467	795	265	30.8	7
4	1215-1763	549	183	20.0	5
5	1763-2362	600	200	22.4	2
6	2353-2871	519	173	19.0	2
7	2864-3247	384	128	13.8	1

Figure 1, which accompanies this description, shows the complete consecutive sequence of the 3383 bp of the cloned fragment corresponding to the 3' end of the PRRS-Olot viral genome. This nucleotide sequence shows 95% homology in comparison with the corresponding sequences of the LV and TV isolates. These two last isolates show, among themselves, 99% homology. The changes in the nucleotide sequence of the PRRS-Olot isolate are found along the entire sequence, but are concentrated especially in 5' end. We should point out, in comparison with LV, the deletion of three nucleotides at position 1860 of PRRS-Olot.

Figure 2 (2A-2F) of this description shows the amino acid

sequences of the proteins coded by ORFs 2 to 7 of the PRRS-Olot virus. At protein level, 99% homology is observed between PRRS-Olot and LV ORF7, as expected for a nucleocapsid viral protein and therefore the more conserved. The percentage of homology for the rest of the proteins ranges between 93% for ORFs 3, 4 and 5 reaching a value of 96.5% for ORFs 2 and 6. All of them present glycosilation sites similar to those described for LV except for ORF4 of the PRRS-Olot virus, which has an extra glycosilation site. With regards to the above-mentioned changes in the PRRS-Olot protein amino acids, 50% of the changes are into chemically similar amino acids, whereas the rest of the changes are into different amino acids. As mentioned for LV, excepting ORF7, the rest of the proteins present a high degree of hydrophobicity, possibly in accordance with their association to membranes since they are viral envelope proteins.

Recombinant proteins corresponding to the expression of PRRS-Olot ORFs 2 to 7 can be produced in a suitable expression system and, advantageously, in an expression system of recombinant baculoviruses multiplied in permissive host cell culture. The global procedure for the obtainment of these recombinant proteins basically comprises the following general stages:

I. Preparation of the cDNA sequence to be inserted into a baculovirus; and

II. Obtainment of recombinant baculoviruses expressing the recombinant proteins.

These general stages are in turn subdivided into other sub-stages. This way, the preparation of the cDNA sequence to be inserted comprises the sub-stages of:

I.a Isolation and purification of the PRRS-Olot virus;

I.b Isolation of the viral RNA of the PRRS-Olot virus; and

I.c Synthesizing of the cDNA from the PRRS-Olot genomic RNA.

On the other hand, the obtainment of recombinant baculoviruses expressing the recombinant proteins corresponding to PRRS-Olot ORFs 2 to 7, comprises the sub-stages of:

II.a Preparation of the PRRS-Olot ORF gene to be inserted;

II.b Inserting of the said gene into a baculovirus transfer vector;

II.c Transfection of permissive host cells with the said transfer vector which has the corresponding PRRS-Olot ORF gene inserted.

II.d Selection of the recombinant baculoviruses expressing the recombinant protein corresponding to the inerted ORF.

The characterization of the recombinant baculoviruses and the analysis and purification of the recombinant proteins are then carried out.

All these stages are described in detail further down in this description.

The procedure employed for the obtainment of the recombinant proteins provided by this invention begins with the isolation and purification of the PRRSV, specifically PRRS-Olot, in accordance with the protocol described in Example 1. Once the PRRS-Olot had been isolated and purified, the viral RNA was isolated and for that purpose a commercial kit (Pharmacia) was used, which makes use of a method based on the selection and purification of the viral RNA containing a poly(A) sequence at 3' end (Example 2). The obtained RNA was analyzed in neutral agarose gels at 0.7% by staining with

ethidium bromide, and only one band of material with molecular weight of between 5000 and 23000 bp was observed.

Afterwards, the cDNA corresponding to the 3' end viral RNA was synthesized (Example 3) with a commercial kit (Boehringer), by means of a strategy which takes advantage of the presence of a poly(A) tail, and uses an oligo d(T) as extension primer capable of being extended with reverse transcriptase enzyme and synthesize cDNA molecules. To clone the 3' upstream RNA regions, an oligonucleotide annealing to a specific viral genome sequence located approximately at 2500 bp from 3' end was used. A second synthesis was carried out using an oligonucleotide of 20 nucleotides instead of the oligo $d(T)_{12}$ (Example 3.1). cDNA synthesis was verified and quantitated by means of counting the radioactivity incorporated in the synthesized material and electrophoresis in alkaline and neutral agarose gels. After this, the cloning and sequencing of the cDNA were carried out (Exmple 3.2). To this end, the first thing done was a size selection of synthesized cDNA fragments of between 1000 and 5000 nt (nucleotides). The purified cDNA was cloned in blunt ends in pMTL25 vector. The analysis of the PRRSV-positive clones was done by means of plasmid DNA preparations and mapping of the restriction sites, based on the LV sequence. Only 9 out of the 300 analyzed plasmids were positive and contained inserts of between 800 and 2600 bp. The definitive verification of the authenticity of these cDNA clones was done by their direct sequencing, using the dideoxys method applied to double-stranded plasmids.

The majority of the obtained positive PRRS clones contained a common poly(A) end and different 5' ends. The clones were denominated as pPRRS-8, pPRRS-108, pPRRS-121, pPRRS-132, pPRRS-146, pPRRS-147, pPRRS-148 and pPRRS-153.

Clone pPRRS-3 was extracted from the second synthesis.

To obtain the recombinant baculoviruses expressing the genes of the proteins coded by PRRSV-Olot ORFs 2 to 7, the following procedure was generally and separately followed: First, the gene from each ORF to be inserted was prepared, except the ORF3 gene which did not require previous preparation. For the preparation of these genes and depending on each particular case, the pMTL25, pMTL24 and pMTL22 plasmids were used before they were transferred into baculovirus transfer vectors. The genes corresponding to ORFs 2 to 7 were obtained from the clones that had been obtained previously. After successive manipulations, they originated new recombinant plasmids. The recombinant plasmids, which contained the genes corresponding to each ORF inserted, were purified following the alkaline lysis technique and were characterized by mapping with restriction endonucleases and sequencing of the insertion regions. The new vectors obtained were denominated as pPRRS-ORFN, where N stands for the number of each ORF (N = 2 to 7).

Then, each ORF gene was cloned into a suitable transfer vector. The transfer vector used was pAcYM1 (Matsuura et al., J. Gen Virol. 68, 1233-50). After successive manipulations, new recombinant plasmids, each one of them containing the inserted ORF gene, were originated. The recombinant plasmids obtained were purified following the alkaline lysis technique and characterized by mapping with restriction endonucleases. The insert ends were sequenced in order to verify correct insert region sequence. The new transfer vectors obtained were analyzed to verify that the inserted genes had the correct orientation for their expression by the AcNPV virus polyhedrin promoter. The transfer vectors obtained were:

Denomination	ORF
pPRRS-Bac8	2
pPRRS-Bac2	3
pPRRS-Bac9	4
pPRRS-Bac3	5
pPRRS-Bac5	6
pPRRS-Bac7	7

Spodoptera frugiperda cells, Sf 9 clone, were then transfected with mixtures of purified infectious DNA of the AcRP23-lacZ parenteral virus and the corresponding transfer vector. Once this transfection had been done, the recombinant baculoviruses were identified by plaque color phenotype assay after the staining of the viral progeny with X-gal, and then purified.

The recombinant baculoviruses obtained were deposited at the European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, Whiltshire SP4 OJG (U.K.).

Examples 4 to 9 describe in detail the obtainment of recombinant baculoviruses expressing the genes coded by ORFs 2 to 7, respectively.

The PRRS-Olot ORF 2 to 7 recombinant proteins can be used with diagnosis purposes to detect the presence of specific PRRSV antibodies (Example 12), and to detect the presence of antigen (PRRSV) by means of antibodies that specifically identify the PRRSV obtained by immunization of animals with, at least, one recombiant protein corresponding to one of PRRS-Olot ORFs 2 to 7. Additionally, these proteins can also be used to immunize animals against PRRSV. Therefore, the said proteins can be used to formulate recombinant vaccines capable of effectively protecting swine against infection caused by PRRSV. These vaccines may be active or passive. Active vaccines can be prepared by suspending at least one of the recombinant proteins provided by this invention in an

immunologically acceptable diluent and an adjuvant. A passive vaccine can be obtained by immunizing animals with the said proteins and isolating the polyclonal antibodies against the said proteins. After antibody isolation and purification, they can be used in vaccine applications.

In a specific realization of this invention, recombinant vaccines are obtained capable of effectively protecting from the infection caused by PRRSV, comprising the viral antigen (antigenic phase) together with an immunologically acceptable diluent and an adjuvant.

For the preparation of the antigenic phase, insect cells —preferentially Spodoptera frugiperda cells— were infected with the diverse recombinant baculoviruses capable of producing the recombinant proteins corresponding to the PRRSV ORFs 2 to 7, and incubated under conditions suitable for the expression of the said proteins. Immediately afterwards, the cells were collected, washed, resuspended in suitable buffer, and then used in the preparation of the aforesaid recombinant vaccines.

In a specific realization, the antigenic phase is composed of a homogenate of insect cells infected with recombinant baculoviruses expressing a single recombinant PRRSV protein, such as, preferently, ORF3, ORF5 and ORF7 (Example 13). In another specific realization, the antigenic phase is composed of a homogenate of a mixture of insect cells infected with different recombinant baculoviruses expressing, each one of them, a different recombinant PRRSV protein, such as a mixture of insect cells infected with the recombinant baculoviruses expressing, for example, the proteins corresponding to ORF3, ORF5 and ORF7.

In general, vaccines were formulated containing as antigenic phase an amount of about $50x10^6$ insect cells infected with baculoviruses expressing the recombinant

protein in question. When the vaccine contains diverse recombinant proteins, the antigenic phase is composed of a quantity of about $50x10^6$ insect cells infected with baculoviruses per the recombinant protein in question, i.e., for a formulation of a vaccine containing the proteins corresponding to ORFs 3, 5 and 7, the antigenic phase is composed of about $50x10^6$ insect cells infected with baculoviruses expressing the ORF3 recombinant protein, $50x10^6$ insect cells infected with baculoviruses expressing the ORF5 recombinant protein, and $50x10^6$ insect cells infected with baculoviruses expressing the recombinant ORF7 protein (Example 13).

Phosphate-buffered saline solutions (PBS) or other similar saline solutions may be used as immunologically acceptable diluents.

As adjuvant, in general, any of the adjuvants habitually used to formulate vaccines may be used, either aqueous —such as aluminum hydroxide, alumina gel suspensions, QuilA— or others, like oily adjuvants, based on mineral oils, glycerides and oleic ether-acid derivatives. In particular, it has been confirmed that an oily adjuvant composed of a mixture of Marcol(R) 52, Simulsol(R) 5100 and Montanide(R) 888, gives very good results. Marcol(R) 52 is a low density mineral oil manufactured by ESSO Española S.A., Simulsol(R) 5100 is a polyethoxy oleate ether commercialized by SEPIC, and Montanide(R) 888 is a high purity anhydromannitol octadecenoate ether commercialized by SEPIC.

The vaccines of this invention can also contain cell response potentiator (CRP) substances, i.e., substances that potentiate helper T cell subpopulations (Th_1 and Th_2) such as IL-1 (interleukin-1), IL-2, IL-4, IL-5, IL-6, IL-12, g-IFN (gamma interferon), cell necrosis factor and similar substances which could, in theory, provoke cell

immunity in vaccinated animals. These CRP substances could be used in vaccine formulations with aqueous as well as oily adjuvants.

Likewise, other types of adjuvants that modulate and immunostimulate cell response can be used, such as MDP (muramyl dipeptide), ISCOM (Immuno Stimulant Complex) or liposomes.

The vaccines of this invention may be obtained by suspending or mixing the antigenic phase with the immunologically acceptable diluent and the adjuvant. When the adjuvant is oily an emulsion is formed which –in a specific and preferred case– if the adjuvant is a mixture of Marcol 52, Simulsol 5100 and Montanide 888 the vaccine will be a double water/oil/water emulsion, type w/o/w.

In the case that the vaccine will contain CRP substances, these substances may be added both to the antigenic phase and to the adjuvant. Alternatively, if the vaccine does not contain any CRP substances, these can be injected, if so desired, simultaneously in a separate site different from the site of inoculation.

Additionally, these vaccines can contain combinations of different porcine pathogens containing, besides one recombinant PRRSV protein or more, one or more of the pathogens mentioned below, allowing for the preparation of polyvalent vaccines. Among these pathogens, but not limited exclusively to them, are Actinobacillus pleuropneumoniae, Haemophilus parasuis, Porcine parvovirus, Leptospira, Escherichia coli, Erysipelothrix rhusiopathiae, Pasteurella multocida, Bordetella bronchiseptica, Porcine respiratory coronavirus, Rotavirus or against the pathogens causative of Aujeszky's disease, swine influenza and transmissible gastoenteritis.

Safety and efficacy trials with the vaccines of the present invention have evidenced that the said vaccines

are safe and at the same time efficacious.

It has been possible to confirm that one dose of 2 ml of a quantity of viral antigen or antigenic phase equal to or higher than $50x10^6$ infected insect cells expressing one or more of the recombinant PRRSV proteins, administered via deep intramuscular route followed by a revaccination with a dose of 2 ml of vaccine, can effectively protect vaccinated animals from the infection caused by PRRSV.

Likewise, it has been possible to verify that some of the vaccines object of the trial –those identified as rPRRS C and rPRRS D– are capable of inducing cellular immunity in vaccinated animals, based on the fact that sows vaccinated and revaccinated with the said vaccines did not present serological at the moment of challenge and, nevertheless, they were protected (Example 14, Tables 4 and 10).

With the purpose of determining and evaluating the efficacy of the prepared recombinant vaccines in the prevention of PRRS in pregnant sows, a trial was designed consisting of the vaccination of pregnant sows with the different vaccines and then submitting them to a discharge test with virulent virus. Based on the obtained results, it has been possible to evaluate the efficacy of the vaccines objective of this trial. In order to evaluate the efficacy of these vaccines, the reproductive results, the number both of piglets alive and dead at different stages of the piglets' life period, as well as the analysis of the serological results in sows and piglets were taken into account (Example 14).

DETAILED DESCRIPTION OF THE INVENTION (EXAMPLES)

Example 1. - Obtainment and purification of the PRRS-Olot virus.

1.1 - Obtainment of pig's lung aleveolar macrophages

1.1.1 -Animals. 7 to 8 week old pigs, a cross between Belgium Landrace and Large White breeds, were used. The animals, from our own farms, were seronegative to the following diseases: Aujeszky's, porcine parvovirosis, foot-and-mouth, classic swine fever, swine influenza (types H1N1 and H3N2) and transmissible gastroenteritis.

1.1.2 -Isolation of macrophages. The animals were anesthetized by injecting in the jugular vein 0.1 g of sodium thiopental per each 10 kg body weight. Then, they were sacrificed and the lungs extracted, after ligating the trachea below the epiglotis and sectioning above the ligation. The extracted lung was washed externally with PBS. Successive internal washings were done (4 to 5) with a total of 500 ml of PBS supplemented with antibiotics at 1:500 (PEG solution: 1000 IU/ml penicillin, 1 mg/ml streptomycin, and 0.5 mg/ml gentamicin), in order to obtain macrophages. These washings were collected together and centrifuged at 300 g for 15 minutes. The following step was to wash the cells twice with PBS by means of consecutive centrifugation/sedimentation, to finally resuspend in DMEMs medium (DMEM supplemented with non-essential amino acids at 100x, GIBCO), containing sodium pyruvate 1 mM, and antibiotics (1:1000 of PEG).

The cells were counted by staining with trypan blue in Newbauer chamber. 0.1 ml of 10^{-1} macrophage suspension was added to 0.4 ml of DMEMs and 0.5 ml of trypan blue solution. In the majority of cases the number of cells obtained ranged between 1 and 1.2 x 10^9.

Sterility controls were carried out on the macrophage

cells by means of seedings in culture media suitable for the detection of bacteria and fungi. Absence of mycoplasma was verified by cytochemical detection with DAPI (4',6-diamidino-2-phenylindole) which selectively attaches to the DNA and forms high specificity DNA-DAPI fluorescent complexes.

1.2 - Replication of the virus in pig alveolar macrophages. Cell culture vials (150 cm^2) were used, containing 100 ml of a macrophage suspension (3 x 10^6 cells/ml) in the DMEMs medium described above, except for the addition of fetal calf serum (FCS) at 5%. The cells were infected with PRRS-Olot virus, isolated by Laboratorios Sobrino and denominated PRRS-JPD-P5-6-91 (ECACC, accession number V93070108). Infection was done at 10^{-3} infection multiplicity, and the infected cells were incubated at 37°C for 24 h. After this period had elapsed, the medium was withdrawn and substituted by fresh DMEMs containing 2% FCS and antibiotics; incubation was continued at 37°C.

The cultures were observed periodically with microscope to determine the cytopathic effect (CPE) produced by the virus on the macrophages. Generally, CPE by 3-4 days of infection was 70-80%. Giant deformed cells appeared. Normally, the titre of these preparations was $10^{6.55}$ $TCID_{50}$/ml (tissue culture infectious dose 50 per milliliter). Macrophages infected at 10^{-4} multiplicity produced viral yields of one order of magnitude less.

The presence of virus in these cells was determined by the immunoperoxidase in monolayer assay on pig macrophage cells obtained as described in Example 1 (1.1.2). Briefly, this was done the following way: In 96-well titration plaques, 100 µl of macrophages were infected with 50 µl of PRRS-OlOT virus replicated on macrophages.

The plaques were incubated for 48 hours at 37°C. Once incubation had been completed, the medium was withdrawn and the plaques washed two times with saline solution (0.1M NaCl). Subsequently, they were fixed with 20% formaldehyde after consecutive incubations at 37°C, -30°C and formaldehyde at 20%. After washing twice with saline solution, 50 µl of a 1:50 dilution of an anti-PRRS serum from a challenged animal. Simultaneous incubations were done with a negative serum from an uninfected animal. Incubation was for 1 hour at 37°C. After withdrawal of the previous solution, they were washed two times with saline solution. Immediately, 0.1 µg of Protein A (Sigma) in 50 µl was added and incubated at 37°C for 1 hour. The assay was developed with AEC (3-amino-9-ethyl-carbazole) dissolved in dimethylformamide in the presence of acetate buffer and oxygenated water. After 15-30 minutes at room temperature in darkness, the plates were observed by microscope. Infected cells appeared stained dark red, in comparison with uninfected cells which were colorless.

1.3 - PRRS virus purification. The virus was purified from PRRSV-infected cell cultures. The culture was clarified by means of centrifugation (20 minutes, 6500 g). The supernatant was concentrated 10X by using a Millipore-Minitan ultrafiltration system (4.5 pSi, 300 kDa pore-size filter). Then, the virus was sedimented by means of centrifugation (5 h, 20000 g). The supernatant was discarded and the precipitate solubilized with PBS containing 1mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) at 4°C, overnight. The virus was purified in discontinuous sucrose gradient (20-50% w/v in PBS) by means of centrifugation at 95000 g for 3 h. Once the centrifugation had been completed, the band containing the virus was extracted from the gradient, diluted with

Tris/EDTA buffer and finally centrifuged overnight at 26000 g for virus sedimentation.

The purified virus was analyzed by means of electrophoresis in polyacrilamide-SDS gels at 12% (Laemmli, U.K., Nature, 227:680, 1970). Total protein was detected by staining with coomassie blue, and immunoblots (Towbin, H., Staehelin, T., and Gordon, J., 1979. Proc. Natl. Acad. Sci. USA, 76: 4350 - 4354). The blots were developed with peroxidase-Protein A (Sigma) conjugate using a covalescent anti-PRRSV serum. It was not possible to observe any specific band related with PRRSV in coomassie-stained gels because of contamination with proteins from the macrophages. However, several viral proteins of molecular weights between 15.5 and 30 KDa were identified by immunoblot. With longer developing times, it was also possible to observe bands of molecular weights over 60 KDa but as these were also detected in uninfected macrophages, it was concluded that they were not PRRS virus-related proteins.

Example 2. - Isolation of the viral RNA

A commercial Pharmacia P-L Biochemicals kit was used.

The method is based on the selection and purification of the viral RNA containing a 3' end poly(A) tail. The viral capsid rupture was done with guanidinium chloride purification of RNA-poly(A) with an oligo-celullose (dT) matrix.

Briefly, the isolation of the PRRS-Olot virus RNA was carried out the following way: The purified virus sedimented by overnight centrifugation at 40000 g. Afterwarads, the supernatant was discarded and the precipitate solubilized with 0.4 ml of the kit extraction buffer. After adsobrption into the cellulose-d(T) matrix, and consecutive washings with the low and high salt

concentration buffers, the RNA-poly(A) was eluted with high ClNa concentration. The RNA was precipitated by adding 1:10 volume of 2.5 M potassium acetate, 0.25 mg/ml glycongen and 2 volumes of ethanol (>2 h. at -20°C). Once this period had elapsed, the RNA was recuperated by centrifugation at 16000 g for 30 minutes After washing the precipitate with ethanol at 75%, it was resuspendend in 20 µl of TE buffer (10 mM Tris-ClH pH=8.0 and 1mM EDTA).

The obtained RNA was analyzed in 0.7% neutral agarose gels by staining with ethidium bromide. A single band of material within 5000 and 23000 bp molecular weight was observed. The absence of low molecular weight material must be pointed out and therefore the possibility of cellular DNA or RNA. However, the amount of material obtained was low – not higher than 100 ng of RNA/250 ml of macrophage culture infected with the virus. This low yield agrees with the low yield of purified virus, as shown by electrophoresis in polyacrilamide gels and electron microscopy (data not shown).

Example 3. - cDNA synthesis from the PRRS-Olot virus genomic RNA

3.1 - Preparation of the cDNA. The cDNA corresponding to the 3' end RNA of the PRRS-Olot viral isolate was synthesized. The strategy takes advantage of the presence of a poly(A) tail in order to use the oligo d(T) as extension primer that can be extended with reverse transcriptase and can synthetize DNA molecule copies. To clone the RNA regions previous to the 3' end, an oligonucleotide with specific sequence of the viral genome located at approximately 2500 bp of the 3' end was used. cDNA synthesis was carried out using a commercial kit (Boehringer). The procedure, in brief, was: 0.1 µg of

PRRS RNA-poly(A), obtained as described in the previous example, was incubated in the presence of 1 mM each dNTPs (dATP, dCTP [5-10 µCi of ^{32}P-α-dCTP], dGTP and dTTP), 25 units of an RNase inhibitor, 0.8 µg oligo $d(T)_{12}$, and 40 units of reverse transcriptase in 20 µl final volume. The reaction was incubated at 42°C for 1 h and then the synthesis of the second strand was startaed in the same tube. To that end, buffer, RNasa, and 25 units of *E. coli* DNA polymerase were added. Incubation was for 1 hour at 22°C, and 10 minutes at 65°C. Finally, to generate blunt ends, 4 units of DNA T4 polymerase were added. After 10 minutes at 37°C, reaction was stopped by adding EDTA and sarkosyl. A second cDNA synthesis was done under the same conditions, except for the fact that 5´CGGGCTCGAGCCTTTGGCGA3´ oligonucleotide was used instead of oligo $d(T)_{12}$. In both cases, the mixture was extracted with phenol:chloroform and the material was precipitated with ethanol, as described in the previous example.

cDNA synthesis was checked and quantified by means of counting the radioactivity incorporated in the synthesized material, and electrophoresis in alkaline and neutral agarose gels.

3.2 - Cloning and sequencing. First, the synthesized cDNA was size selected to avoid the cloning of excessively small segments. For that purpose, the material from the cDNA synthesis was recovered by centrifugation (30 minutes, 16000 g). The precipitate was vacuum dried, dissolved with Tris/EDTA buffer (TE) pH=8.0, and loaded in 1% agarose gel. The cDNA fragments between 1000 and 5000 bp were recovered from the gel with DEAE-cellulose paper and from the latter by elution with ClNa and subsequent precipitation. Purified cDNA was cloned in blunt ends in the pMTL25 vector, a vector derived from the pUC18. With

that purpose, the vector was linearized with *SmaI* and treated with alkaline phosphatase to reduce the vector background. After ligation with DNA T4 ligase, *E.coli* XL-1Blue competent cells were transformed with the ligation mixture in the presence of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Boehringer) and IPTG (Isopropyl β-D-thiogalactopyranoside) (Gold Bioch), which allows the initial selection of recombinant colonies by color (blue colonies without insert in comparison with white ones with insert).

The analysis of the positive PRRS clones was done by means of plasmid DNA preparations (Birnboim & Doly, Nucleic Acids Res., 7, 1513-1523, 1979), and mapping of restriction sites based on LV sequence. Only 9 out of the 300 plasmids analyzed were positive and contained inserts between 800 and 2600 bp. The definitive verification of the authenticity of these cDNA clones was done by their directe sequencing, using the dideoxy chain-termination method applied to double-strand DNA (Sanger, F., et al., J. Mol. Biol., 94:441-448, 1975). The universal oligonucleotides (5'GTAAAACGACGGCCAGT3') and reverse (5'AACAGCTATGACCATG3') oligonucleotides were used to sequence all the clones. The majority of the obtained PRRS clones contained one common poly(A) tail and different 5' ends. The clones were denominated pPRRS-8, pPRRS-108, pPRRS-121, pPRRS-132, pPRRS-146, pPRRS-147, pPRRS-148 y pPRRS-153. From the second cDNA synthesis, clone PRRS-3 was obtained. Figure 3 shows the different extension of these clones in comparison with LV, as well as the ORFs contained in each one. On the other hand, Figure 1 shows the consecutive sequence of the 3383 bp cloned from the PRRS-Olot isolate, and Figure 2 (2A-2F) shows the amino acid sequences corresponding to the proteins coded by each ORF.

Example 4. - Obtainment of recombinat baculoviruses expressing the protein gene coded by ORF2

4.1 - Preparation of the ORF2 gene

The pMTL25, pMTL24 y pMTL22 genes, derived from the pUC18 vector, were used for the preparation of the different ORFs mentioned in this description, before they were cloned in baculovirus transfer vectors. The vector used is indicated for each particular case. The ORF2 gene is 747 bp in size, and was obtained from cDNA pPRRS-3 clone (Figure 4). The DNA was digested with *MaeI*, and the insert of approximately 900 bp was purified in agarose gel. The cohesive insert ends were transformed into blunt ends by means of treatment with the Klenow fragment of the *E. coli* DNA polymerase. Cloning was done in the pMTL25 treated with *SmaI*, alkaline phosphatase and purified in 1% low melting agarose gel. After ligation with DNA T4 ligase (Boehringer), *E.coli* XL-1Blue cells were transformed with the ligation mixture and the positive clones selected initially by color. The recombinant plasmids containing the inserted ORF2 gene were purified according to the alkaline lysis method (Birnboim & Doly, Nucleic Acids Res., 7, 1513-1523, 1979), and characterized by mapping with restriction endonucleases and sequencing of the insertion regions.

The newly obtained vector was denominated pPRRS-ORF2. In it, the ORF2 initiation codon (ATG) is located approximately at 50 bp from the beginning of the insert and the *BamHI* site.

4.2. - Insertion of the ORF2 gene into a baculovirus transfer vector

The baculovirus transfer vector used in all the experiments, described in this patent claim, was pAcYM1

vector (Matsuura et al., J. Gen Virol. 68, 1233-50), which has a single *BamHI* insertion site.

The vector was donated by Professor D.H.L. Bishop (I.V.E.M., Oxford, United Kingdom). For the insertion, the vector was thoroughly digested with the *BamHI* endonuclease and then treated with the alkaline phosphatase enzyme to avoid vector religation. ORF2 codes for a 28.4 KDa protein. Briefly, the insertion of the corresponding gene into the pAcYM1 vector used pPRRS-ORF2 plasmid as a starting material. In this plasmid, the ORF2 gene is flanked by two *BamHI* sites. Thus, the pPRRS-ORF2 is digested with *BamHI* and loaded in 1% low melting agarose gel in order to obtain the 935 bp fragment. This fragment was inserted into the *BamHI* site of pAcYM1 according to Struhl's method (Biotechniques 6, 452-453, 1985), using the DNA T4 ligase (Boehringer) to ligate the insert the vector. The ligation mixture was used to transform *E. coli* DH5 cells. The obtained recombinant plasmids containing the inserted ORF2 gene were purified according to the alkaline lysis method (Birnboin & Doly, supra), characterized by mapping with restriction endonucleases and sequenced the insert edges to corroborate the correct sequence of the insertion regions. The newly obtained transfer vector was denominated **pPRRS-Bac8** and it was shown to have the PRRS gene in the correct orientation for its expression by the AcNPV baculovirus polyhedrin promoter.

4.3 - Transfection and selection of baculoviruses

Spodoptera frugiperda cells, Sf 9 clone, were cotransfected with a mixture of purified infective DNA of parental virus AcRP23-lacZ (500 ng), donated by Dr. Posee (I.V.E.M., Oxford, U.K.) and the transfer vector **pPRRS-Bac8** DNA (2 µg). The parental virus DNA was linearized

with the *Bsu36I* enzyme within the *lacZ* gene (Kitts et al., Nuc. Acids Res. 18, 5667-72.1990) in order to increase the efficiency of the recombination. For cotransfection, the lipofectin (Gibco-BRL) method was used (Felgner et al., Proc. Natl. Acad. Sci. U.S.A., 84, 7413-7417, (1987)). After cotransfection, the cells were incubated for 5 days in complete TNMFH medium supplemented with 5% fetal calf serum (FCS) and antibiotics, until cytopathic effect was observed.

Then, the transfection supernatant was recovered and the recombinant viruses identified by plaque assay. The AcRP23-*lacZ* parental virus shows blue lysis plaques in the presence of X-gal substrate because the β-galactosidase gene is being expressed. Recombinant viruses were initially identified by the clear plaques after staining the viral progeny with X-gal. A number of plaques of each virus were picked and subjected to three purification rounds, before a high titre virus stock was prepared. The recombinant baculovirus finally obtained was denominated **AcNPV, PRRS 2.** It has been deposited at the European Collection of Animal Cell Cultures (ECACC) with accession number V94021007.

Example 5 - Obtainment of recombinant baculoviruses expressing the protein gene coded by ORF3

5.1 - Insertion of ORF3 gene into a baculovirus transfer vector

ORF3 codes for a protein of an estimated molecular weight of 30.8 KDa. pPRRS-121 plasmid DNA was used as a starting material for the insertion of the corresponding gene in the pAcYM1 transfer vector (Figure 5). In this vector, the ORF3 initiation codon is located 10 bp from the *BamHI* site. The gene can be excised by double digestion with the *BamHI* and *Sau3A* enzymes, which generates cohesive ends

compatible with *BamHI*. After digestion, the mixture was loaded in 1% low melting agarose gel, and a 1009 bp fragment was purified. It was isolated and then ligated to the pAcYM1 vector treated with *BamHI* and alkaline phosphatase, using the T4 ligase DNA enzyme. Subsequently, *E. coli* DH5 cells were transformed and the recombinant plasmids purified and characterized according to the procedures described above. Once the correct sequence and insert orientation towards the polyhedrin promoter had been verified, the new transfer vector was denominated **pPRRS-Bac2**.

5.2 - Transfection and selection of recombinant baculoviruses

The procedure used for the transfection and selection of recombinant baculoviruses was similar to the one described above for ORF2 (Example 4.3). The recombinant baculovirus obtained was denominated **AcNPV, PRRS 3**. It has been deposited at ECACC with accession number V94011325.

Example 6. - Obtainment of recombinant baculoviruses expressing the protein gene coded by ORF4

6.1 - Preparation of the ORF4 gene

The size of the ORF4 gene is 549 bp. It was obtained from the pPRRS-146 clone (Figure 6) digested with the *BamHI, AflIII* and *PstI* enzymes. The first two enzymes flank the insert and *PstI* was used to cleave a vector DNA fragment, of similar size to the ORF4 gene which would have made gene isolation difficult. A 1112 bp fragment was purified in low melting agarose gel and cloned in pMTL22 vector digested with *BamHI* and *NcoI* (compatible with *AflIII*). After ligation with T4 ligase DNA and transformation of *E. coli* DH5 cells, the recombinant plasmids were purified

accoding to the alkaline lysis method (Birmboin & Doly, supra), and characterized by restriction endonuclease mapping. The newly obtained vector was called **pPRRS-ORF4**. It contains the ORF4 initiation ATG codon located 5 bp from the *BamHI* site.

6.2 - Insertion of the ORF4 gene in a baculovirus transfer vector

ORF4 codes for a 20.0 KDa protein. The corresponding gene was obtained from the pPRRS-ORF4 plasmid by digestion with *BamHI* plus *BglII*. The 1112 bp fragment was purified in 1% low melting agarose gel and directly cloned in pAcYMI-*BamHI*. The procedures for the identification and characterization of the recombinant clones were identical to those described above (Example 4.2). Once the correct orientation and insert sequence had been verified, the new plasmid was denominated **pPRRS-Bac9**. This plasmid was used for posterior transfection experiments and preparation of recombinant baculoviruses.

6.3 - Transfection and selection of reombinant baculoviruses

The procedure followed for the transfection and selection of recombinant baculoviruses was similar to the procedure described above for ORF2 (Example 4.3). The recombinant baculovirus was denominated **AcNPV, PRRS4**. It has been deposited at ECACC with accession number V94021008.

Example 7. - Obtainment of recombinant baculoviruses expressing the protein gene coded by ORF5

7.1 - Preparation of the ORF5 gene

The size of ORF5 is 600 bp. It was obtained from clone pPRRS-132 (Figure 7). The DNA was digested with the *BstXI* and *BfrI* enzymes, and a 700 bp fragment containing ORF5

was purified in 1% low melting agarose gel. After converting the fragment ends from cohesive to blunt by means of treatment with T4 polymerase DNA, the fragment was cloned in the pMTL25/*SmaI* vector. The method used was similar to the procedures described in Example 4.1. The newly obtained vector was denominated **pPRRS-ORF5**. It contains the ORF5 initiation ATG codon, located 15 bp from the beginning of the gene.

7.2 - Insertion of the ORF5 gene in a baculovirus transfer vector

ORF5 codes for a 22.4 KDa protein. To insert the corresponding gene in the transfer vector, the pPRRS-ORF5 vector was digested with enzyme *BamHI*. The 706 bp fragment was purified in 1% low melting agarose gel and ligated directly to the pAcYm1-*BamHI* transfer vector. The recombinant plasmids were characterized as described above. The new transfer vector was denominated **pPRRS-Bac3**. It was used in subsequent transfection experiments.

7.3 - Transfection and selection of recombinant baculoviruses

The procedure followed for the transfection and selection of recombinant baculoviruses was similar to the procedure described above for ORF2 (Example 4.3). The recombinant baculovirus obtained was denominated **AcNPV, PRRS5** and has been deposited at ECACC with accession number V94011326.

Example 8. - Obtainment of recombinant baculoviruses expressing the protein gene coded by ORF6

8.1 - Preparation of the ORF6 gene

The size of the ORF6 gene is 519 bp. It was prepared from the pPRRS-8 gene clone (Figure 8). First, the DNA was digested with the *AflIII* enzyme, which allowed the

elimination of bands approximate in size to the ORF6 gene. A 990 bp *AflIII-AflIII* fragment was purified in 1% low melting agarose gel and digested with *TaqI*. The new 790 bp fragment was purified in low melting agarose gel and cloned in the pMTL24 vector treated with *AccI* and alkaline phosphatase. Subsequently, the steps described in Example 4.1 were done. The new vector was denominated **pPRRS-ORF6**. It contains the ORF6 initiation codon located at 46 bp from the beginning of the gene.

8.2 - Insertion of the ORF6 gene in a baculovirus transfer vector

ORF6 codes for a 19.0 KDa protein. This is supposed to be the envelope protein and, on account of its hydrophobic nature, it is considered to be a membrane-spanning protein. For the insertion of the corresponding gene in the transfer vector, the pPRRS-ORF6 vector, containing the ORF6 gene cloned at pMTL24 *AccI* site, was digested with the *BamHI* enzyme. The 790 bp fragment was purified from the 1% agarose gel and ligated directly to vector pAcYM1-*BamHI*. The new transfer vector was denominated **pPRRS-Bac5**. It was used in subsequent transfection experiments.

8.3 - Transfection and selection of recombinant baculoviruses

The method used for the transfection and selection of recombinant viruses was similar to the procedure described above for ORF2 (Example 4.3). The recombinant baculovirus obtained was denominated **AcNPV, PRRS6**. It has been deposited at the ECACC with accession number V94011327.

Example 9. - Obtainment of recombinant baculoviruses expressing the protein gene coded by ORF7

9.1 - Preparation of the ORF7 gene

The size of the ORF7 gene is 384 bp. It was prepared from the pPRRS-8 gene clone (Figure 8). Fragment *AflIII-AflIII* described in Example 8.1 was digested with the *HpaI* enzyme. The 430 bp *AflIII-HpaI* fragment containing the ORF7 gene was purified in low melting agarose gel and subsequently cloned in the pPMTL25 vector digested with *NcoI-SmaI*. The analysis and characterization of recombinant colonies was done as described in Example 4.1. The new vector was denominated **pPRRS-ORFT**. It contains the ORF7 initiaiton codon located at 16 bp from the beginning of the gene.

9.2 - Insertion of the ORF7 gene in a baculovirus transfer vector

ORF7 codes for a 13.8 KDa protein. This is supposed to be the viral nucleoprotein. For the insertion of the corresponding gene in the transfer vector, the pPRRS-ORF7 plasmid was digested with the *BglII* and *BamHI* enzymes. The resulting 430 bp fragment was isolated from a low melting agarose gel and ligated directly within the pAcYM1-*BamHI* vector. After the suitable characterizations, the new **pPRRS-Bac7** transfer vector was obtained. It was used in subsequent transfection experiments.

9.3 - Transfection and selection of recombinant baculoviruses

The method used for the transfection and selection of recombinant baculoviruses was similar to the procedure described above for ORF2 (Example 4.3). The recombinant baculovirus obtained was denominated **AcNPV, PRRS7**. It has

been deposited at the ECACC with accession number V94011328.

Example 10. Analysis of recombinant proteins and immunodetection

Sf9 cells were infected with different recombinant baculoviruses at multiplicity of infection of 1 PFU/cell and incubated at 27°C until the cultures were harvested. Different cell cultures were done in monolayer and in suspension. In all the cases, results were similar. The cultures were harvested at different post-infection times. The optimal harvesting time for each recombinant virus was determined. This ranged from between 48 and 96 p.i.h. (post-infection hours). The cells were harvested by centrifugation at 1500 rpm for 10 min, washed twice with PBS pH:7.4 and subsequently resuspend and lysed with 25mM bicarbonate solution. They were centrifuged at 10000 rpm for 10 minutes and the soluble cytoplasmic fraction was separated from the remaining insoluble cell debris. The total cell extracts as well as the different fractions were analyzed by electrophoresis in 11% polyacrilamide gels and stained with coomassie blue or transferred to nitrocellulose membranes for immunological detection. Bands were observed by staining with coomassie blue with molecular weights of 28.4, 30.8, 20.0, 22.4, 19.0 and 13.8 KDa. These sizes correspond respectively to the sizes expected for the genes coded by ORFs 2, 3, 4, 5, 6 and 7. There is a significant variation in the expression levels of the different genes: ORFs 3, 5 and 7 at considerable level, ORFs 2 and 4 at appreciable level and ORF6 at low level. The genes lower expression levels, corresponidng to ORFs 2 and 6, might be due to the larger distance, 42 and 39 nucleotides respectively, between the protein initiation ATG codon and the polyhedrin baculovirus

promoter. On several occasions, it has been demonstrated that this distance should essentially be maintained at a minimum in order to obtain a good expression. Another factor, responsible for low expression, could be the high hydrophobic nature of these proteins.

When analyzing separately the soluble and insoluble fractions of the infected cells, it has been observed that, except for ORF7, most of the expressed PRRS proteins are insoluble and remain associated to the membrane debris. This may be due to the hydrophobic and glycosilated nature of these proteins. The majority of these glycoproteins contain transmembrane regions that anchor them to the membranes. Such characteristics make the purification of these proteins from cell extracts difficult.

For immunodetection, the proteins were transferred to nitrocellulose membranes, according to standard methods (Burnette, Anal. Biochem. 112, 195-203, 1981; Towbin et al., Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354, 1979). Protein transfer was done in a semi-dry device (Bio-Rad) at 22V for 30 minutes. Then, the nitrocellulose strips were blocked with 3% powder skim milk in Tris-HCl 20mM pH 7.5, NaCl 500 mM (TBS) for 1 hour at room temperature. Subsequently, the strips were incubated first for two hours at room temperature with an anti-PRRS pig antiserum (C-45) diluted 1/100 in TBS-0.05% Tween 20, washed with TBS-0.05% Tween 20 for 30 minutes at room temperature, and then incubated with anti-pig IgG conjugated to alkaline phosphatase (dilution 1/1000) (Sigma) for 1 hour. The strips were washed once more and, finally, developed with an NBT (nitro blue tetrazolium) (Sigma) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Sigma) solution in NaCl 100 mM, $MgCl_2$ 5 mM, diethanolamine 100 mM, pH: 9.5, until the appearance of visible bands. The reaction was stopped

with distilled water. In all the cases in which specific reactions were seen by immunoblot, proteins of molecular weight equivalent to the estimated ORF sizes were obtained. In some cases, specifically in ORFs 3 and 5, the presence of other larger-sized bands, till 45 KDa, were observed. These bands would represent different protein glycosilation forms, in agreement with the foreseen potential sites.

10.1 - Antigenic characterization of the recombinant proteins

The correct antigenicity of the recombinant proteins expressed in baculovirus was checked by their reaction to different animal sera in an immunoblotting assay.

Recombinant proteins expressed and transferred to nitrocellulose according to the above method, were made to react with a collection of previously characterized swine sera containing anti-PRRSV antibodies. The sera had been obtained in animals infected experimentally (#1-4) or naturally (#5-8).

Proteins corresponding to ORFs 3, 5 and 7 were the first to be checked. Results are shown in Table 2.

Table 2

Reactivity of sera from infected animals against ORF3, ORF5 and ORF7 recombinant proteins

Serum no.	ORF3	ORF5	ORF7
1	+	+	-
2	+	+	-
3	+	+	+
4	ND	+	+
5	ND	+	+
6	+	+	+
7	ND	+	-
8	ND	+	+

+: Positive
-: Negative
ND: Not determined

This assay demonstrated that recombinant proteins 3, 5 and 7 are antigenically similar to native viral proteins 3, 5 and 7, respectively.

When the assay was done with recombinant proteins 2, 4 and 6, the results were of a greater variability in what respects recognition by field sera. The reasons for this variability may be their low expression level and/or their high hydrophobicity.

These assays demonstrate that PRRSV recombinant proteins expressed in baculovirus system are not antigenicallly distinguishable from native viral proteins.

Example 11. Purification of the recombinant proteins

The strategy designed for recombinant protein purification

should take into consideration the structural characteristics of the proteins. Two of these characteristics should be pointed out:

(1) hydrophobic nature which makes them insoluble, and (2) presence of a large number of transmembrane regions which gives them a great affinity to membranes. In most cases, these characteristics do not make protein extraction and purification convenient, e.g.: for their use as a vaccine, when complete infected cells can be used, as described by different authors (Hall S.L., et al., Vaccine, 9, 659-667, Sept. (1991); Tordo N., et al., Virology, 194, 5269 (1993)). In spite of this, some attempts have been made to purify these proteins using ORF3 protein as a model.

11.1 - Purification of the protein derived from ORF3

Sf9 cells were infected with the recombinant **AcNPV, PRRS3** virus, according to the method described in the previous Example. The infected cells were collected by centrifugation at 400 g for 10 min, washed with PBS and resuspended at $20x10^6$ cells/ml in PBS. The cells were disrupted by freezing/thawing and the soluble fraction was separated from the insoluble fraction by centrifugation. In all the cases, the insoluble fraction was used for the subsequent treatments.

Below is a description of some of the methods used:

Treatment with chaotropic agents

The insoluble fraction was first washed with 1M NaCl and then with 2M or 4M guanidinium chloride. The cell pellets were resuspended in the different buffers and maintained at room temperature for 1 hour. Then, the preparation was centrifuged at 15000 rpm for 5 minutes The presence of the recombinant protein in the different fractions was analyzed by elecrophoresis in 15% polyacrylamide-SDS gels (sodium dodecyl sodium sulfate).

The results obtained indicate that the sequential treatment with these salts yields a protein of 30% to 50% purity. This purified protein has been shown to be antigenically analogous to native protein, as it is recognizable by sera from infected animals, determined either by immunoblotting or indirect ELISA.

Treatment with detergents

Detergents at the following concentrations were used:

-NP40	0.5%
-Octylglucoside	2%
-SDS	0.5%, 1% and 2%
-Sodium deoxycholate	0.5%, 1% and 2%

In all cases the cell preparations were done analogous to the one described above. Cell debris containing recombinant protein were treated with the above detergent concentrations and under the described conditions. In general, it can be stated that under these conditions, treatment with the different detergents did not enable the solubilization of a significant amount of recombinant protein. Only 0.5% SDS yielded protein of 50% estimated purity, although with very low yield. Antigenically, this protein reacts with infected animal sera by direct ELISA, although the efficacy is lower than what is obtained with the protein purified with chaotropic agents.

To summarize, these partially purified proteins could be used in anti-PRRSV vaccines.

Example 12. Diagnostic use

One of the main applications of the recombinant proteins provided by this invention is their use in the preparation of kits for the diagnosis of PRRSV field infections.

12.1 - Preparation of antigen expressed in Sf9 for application in diagnosis.

Sf9 cells grown in monolayer or in suspension were infected at multiplicity of infection of 0.5 to 1 with the respective recombinant baculoviruses. Depending on which recombinant virus was used, cultures were harvested between 48 and 72 hours post infection. They were centrifuged at 400 g at 15°C for 10 minutes and washed with PBS.

Finally, the cell pellets containing the recombinant proteins were resuspended in PBS with 2% octylglucoside (Sigma) and were allowed to stand on ice for 1 hour. They were then centrifuged at 1000 g for 10 minutes to eliminate cell debris. The supernatants were exhaustively dialyzed against PBS to remove the detergent, centrifuged at 10000 g for 30 minutes to remove precipitates and stored at -70°C until later use.

12.2 - ELISA for diagnosis.

Polystyrene 96-well ELISA immuno plates (Polisorp, NUNC) were coated with different dilutions of the recombinant extracts mixture (ORF2, ORF3, ORF4, ORF5, ORF6 and ORF7), made in 50 mM carbonate buffer pH:9.6 (100 µl/well) by overnight incubation at 4°C. As shown in Figure 9, the optimal dilution chosen for the plate coatings was 1/100. The plates were saturated with blocking buffer (1% skim milk in PBS) for 30 minutes at room temperature. Subsequently, were added different dilutions of the anti-PRRSV antisera made in blocking buffer. Incubation was continued for 1 hour at 37°C. After washing with PBS containing 0.05% Tween 20, peroxidase-labeled protein A (1/5000 dilution) was added, incubating at 37°C for 1 hour. A washing like the previous one was done and the reaction was developed at room temperature for 10 minutes

using ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6 sulfonic acid)] as substrate. The reaction was stopped with 1% SDS and absorbance was monitored at 405 nm.

Usual ELISA titration results from an infected animal field serum are shown on Figure 10. Field sera titrations normally range from 1/100 to 1/800 dilutions. The results obtained in a sampling experiment with several dozen field sera are shown on Figure 11. It can be seen that titres obtained for clearly positive sera range from 0.4 to 1.7. Titres from uncertain sera range from 0.2 to 0.3. Negative sera give titres under 0.1. Thus, the conclusion arrived at is: the use of these recombinant proteins expressed in baculovirus is a safe, reliable and reproducible method, which enables to conclusively differentiate infected from uninfected animals.

Example 13. Formulation of the recombinant vaccines.

Diverse vaccines were prepared containing different recombinant PRRSV proteins, specifically PRRS-Olot [ECACC V93070108] in emulsion form, in accordance with the method described below.

Spodoptera frugiperda cells, clone Sf9 –hereunder Sf9– were infected at the rate of $1x10^6$ cells/ml with the recombinant baculoviruses:

- AcNPV, PRRS3, [ECACC V94011325];
- AcNPV, PRRS5, [ECACC V94011326]; and
- AcNPV, PRRS7, [ECACC V94011328],

capable of producing, respectively, the recombinant proteins corresponding to ORF3, ORF5 and ORF7 of the aforesaid PRRSV (Figures 2, 4 and 6), at infection multiplicity of 0.1 plaque forming units (PFU)/cell. They were incubated at 27°C, with stirring at 100 rpm and 30% of pO_2, for 72 hours, in a 2 liter Braun-MD fermentor. Then the infected insect cells were collected by centrifuging

at 1000 rpm for 10 minutes, washed with phosphate buffered saline solution (PBS) pH:7.4 and suspended at $5x10^7$ cells/ml in the same PBS buffer.

The vaccines were formulated by mixing an infected Sf9 cell homogenate containing $50x10^6$ Sf9 cells expressing each one of recombinant proteins ORF3, ORF5 and ORF7, with an oily adjuvant, or oily phase, composed of a mixture of:

Marcol(R) 52.....790.0 mg
Simulsol(R) 5100..... 70.0 mg
Montanide(R) 888..... 80.0 mg

Under these conditions, 4 recombinant vaccines were prepared, in doses of 2 ml, composed of 53% antigenic phase and 47% of the oily phase described above, in which the oily phase/antigenic phase relation is a weight/volume relation (W/V). The prepared vaccines presented the following formulation:

1. Vaccine identified as rPRRS C:
   53%, by volume, of antigenic phase composed of $50x10^6$ Sf9 cells expressing ORF3; and
   47%, by weight, of the oily phase as described above.
2. Vaccine identifed as rPRRS D:
   53%, by volume, of antigenic phase composed of $50x10^6$ Sf9 cells expressing ORF5; and
   47%, by weight, of the oily phase as described above.
3. Vaccine identified as rPRRS E:
   53%, by volume, of antigenic phase composed of $50x10^6$ Sf9 cells expressing ORF7; and
   47%, by weight, of the oily phase as described above.
4. Vaccine identified as rPRRS F:
   53%, by volume, of antigenic phase composed of $50x10^6$ Sf9 cells expressing ORF3; $50x10^6$ Sf9 cells expressing ORF5, and $50x10^6$ Sf9 cells expressing ORF7, (total $150x10^6$ Sf9 cells); and

47%, by weight, of the oily phase as described above.

Example 14. Efficacy in pregnant sows

This trial was carried out to evaluate the efficacy of the recombinant vaccines prepared as described in Example 13. To that end, a total of 12 sows —a Landrace X Large White cross— was used. The animals were transferred to the safety stables of the research center.

Two sows were chosen at random (sows no. 400398 and 400298) and were vaccinated with the vaccine identified as rPRRS C. Two sows (sows no. 400118 and 400307) were vaccinated with the vaccine identified as rPRRS D. With the vaccine identified as rPRRS E three sows were vaccinated (sows no. 314010, 313426 and 400059), and with the vaccine identified as rPRRS F three sows were vaccinated (sows no. 313524, 401236 and 401426). The two remaining sows (sows no. 1 and 20) were not vaccinated and were used as control animals.

The sows were vaccinated via deep intramuscular route (IM) in the neck, close to the ear, with a dose of 2ml of vaccine, and revaccinated 21 days later with the same dose.

Local and general reactions were observed, such as: rectal temperature, feed intake and clinical signs both post-vaccination and post-challenge. Additionally, reproductive post-challenge results in the sows were monitored, as well as the serological results both in sows and piglets. The analysis of the results was used in the evaluation of the efficacy of the vaccine (Table 1).

Challenge was done in the safety stables of the research center. All the animal were infected at the rate of 5 ml of PRRSV-218-P6-MΦ-F22055-29/10/94, a strain isolated and maintained at the deposits of the research center, with a

titer of $10^{6.1}TCID_{50}$/ml (tissue culture infectious dose 50%) via intranasal route (IN).

For the evaluation of the sows' reproductive results on the day of farrowing, the following data were noted down (Table 3):

- no. of piglets born alive and in good health
- no. of piglets born alive but weak
- no. of stillborn piglets
- no. of piglets with partial autolysis (edematous)
- no. of mummified piglets
- piglets alive after the 1st week of life, and
- piglets alive at the time of weaning (25-30 days of age).

Table 3

Reproductive results

SOW No.	VACCINE	NUMBER OF PIGLETS TOTAL	BORN ALIVE HEALTHY	BORN ALIVE WEAK	STILL-BORN	PARTIAL AUTOLYS.	MUMMI-FIED	PIGLETS ALIVE 1st WEEK	PIGLETS WEANED
1	CONTROL	17	-	4	9	4	-	-	-
20	CONTROL	14	9	-	2	3	-	7	4
400398	rPRRS C	8	8	-	-	-	-	7	6
400298	rPRRS C	11	10	1	-	-	-	8	7
400118	rPRRS D	12	6	1	2	3	-	5	- 4
400307	rPRRS D	10	9	-	1	-	-	9	7
314010	rPRRS E	12	-	10	1	1	-	3	2
313426	rPRRS E	6	3	-	-	1	2	3	3
400059	rPRRS E	12	6	2	2	2	-	1	0
313524	rPRRS F	11	10	-	1	-	-	10	8
401236	rPRRS F	2	-	-	-	-	-	2	2
401426	rPRRS F	15	12	3	-	-	-	10	10

Then, serological response was analyzed in the sows (Table 4) and piglets (Tables 5, 6, 7, 8 and 9) by means

of a peroxidase monolayer assay (IPMA) [Immuno Peroxidase Monolayer Assay, Wensvoort et al., Vet. Quaterly, Vol. 13, nº 3 (July 1991)], in accordance with the following program:

D 0 (Day 0): Bleeding and vaccination

D + 14: Bleeding [at 14 days post-vaccination]

D + 21: Bleeding and revaccination [21 days post-vaccination]

D + 28: Bleeding [28 days post-vaccination]

D + 35: Bleeding [35 days post-vaccination]

D I: Bleeding and challenge

D I+7: Bleeding [at 7 days post-infection]

Serological results in the sows (anti-PRRSV antibodies) are shown in Table 4.

Table 4

Serological results (anti-PRRSV antibodies)

Vaccine	Sow	D 0	D+14	D+21	D+28	D+35	D I	D I+7
rPRRS C	400298	--	320	320	NT	160	320	≥640
rPRRS C	400398	--	--	--	NT	--	--	≥640
rPRRS D	400307	--	--	--	--	--	--	≥640
rPRRS D	400118	--	--	--	--	--	--	≥640
rPRRS E	314010	--	≥640	≥640	≥640	≥640	160	320-640
rPRRS E	313426	--	≥640	≥640	≥640	≥640	320	≥640
rPRRS E	400059	--	≥640	320	NT	≥640	≥640	≥640
rPRRS F	313524	--	320-640	320	≥640	≥640	320-640	≥640
rPRRS F	401236	--	≥640	≥640	≥640	≥640	≥640	320
rPRRS F	401426	--	320	NT	NT	320	160	≥640
CONTROL	1	--	NT	NT	NT	NT	--	160
CONTROL	20	--	NT	NT	NT	NT	--	80

[NT: Not tested; --:Negative]

Table 5

Serological results obtained in the piglets born to control animals (unvaccinated)

SOW No.	BEFORE WEANING				WEANING		POST-WEANING			
	No.	AGE DAYS	REF	Ab	No.	AGE DAYS	No.	AGE DAYS	REF	Ab
1	2	2	1	≥640	0	-	0	-		
			2	≥640						
20	7	12	436	320	4	33	3	39		
			437	320					437	320
			438	320					438	320-640
			439	≥640						
			440	160						
			441	320-640					441	≥640
			442	≥640						

Sow No.: Reference of the sow

No.: Number of piglets; Ab: Antibodies; -: Negative

REF: Reference of the piglet

Tabla 6

Serological results obtained in the piglets born to animals vaccinated with rPRRS C (ORF3)

SOW No.	BEFORE WEANING				WEANING		POST-WEANING			
	No.	AGE DAYS	REF	Ab	No.	AGE DAYS	No.	AGE DAYS	REF	Ab
400398	8			N.T.	7		6			N.T.
400298	8	7	482	160	7	28	6	42	482	-
			483	160					483	-
			484	≥640					484	N.T.
			485	320-640					485	-
			486	≥640					486	-
			487	320					487	-
			488	80						
			489	160						

Sow No.: Reference of the sow

No.: Number of piglets; Ab: Antibodies; N.T.: Not tested; -: Negative

REF: Reference of the piglet

Table 7

Serological results obtained in the piglets born to animals vaccinated with rPRRS D (ORF5)

SOW No.	BEFORE WEANING				WEANING		POST-WEANING			
	No.	AGE DAYS	REF	Ab	No.	AGE DAYS	No.	AGE DAYS	REF	Ab
400118	5	9	415	≥640	4	30	3	44		
			416	80						
			417	320					417	-
			418	80-160					418	-
			419	160					419	-
400307	9	4	424	160	7	25	7	30	424	-
			425	≥640					425	-
			426	≥640					426	-
			427	-					427	N.T.
			428	160					428	-
			429	320-640					429	80-160
			430	-						
			431	160						
			432	≥640					432	-

Sow No.: Reference of the sow

No.: Number of piglets; Ab: Antibodies; N.T.: Not tested; -: Negative

REF: Reference of the piglet

Table 8

Serological results obtained in the piglets born to animals vaccinated with rPRRS E (ORF7)

SOW No.	BEFORE WEANING				WEANING		POST-WEANING			
	No.	AGE DAYS	REF	Ab	No.	AGE DAYS	No.	AGE DAYS	REF	Ab
314010	2	10	411	80	2	31	1	45		
			412	320					412	160
313426	3	2	421	≥640	3	30	3	37	421	-
			422	≥640					422	320
			423	≥640					423	160
400059	4	3	1	N.T.	0					
			2	N.T.						
			3	N.T.						
			4	N.T.						

Sow No.: Reference of the sow

No.: Number of piglets; Ab: Antibodies; N.T.: Not tested; -: Negative

REF: Reference of the piglet

Table 9

Serological results obtained in the piglets born to animals vaccinated with rPRRS F (ORF3+5+7)

SOW No.	BEFORE WEANING				WEANING		POST-WEANING			
	No.	AGE DAYS	REF	Ab	No.	AGE DAYS	No.	AGE DAYS	REF	Ab
313524	10	10	401	≥640	8	30	8	45	401	≥640
			402	≥640					402	≥640
			403	80-160						
			404	≥640					404	≥640
			405	≥640					405	≥640
			406	≥640					406	≥640
			407	≥640					407	320
			408	≥640					408	≥640
			409	≥640					409	≥640
			410	≥640						
401236	2	7	413	≥640	2	27	2	42	413	80
			414	≥640					414	80

Sow No.: Reference of the sow

No.: Number of piglets; Ab: Antibodies

REF: Reference of the piglet

Table 9 (Cont.)

Serological results obtained in the piglets born to animals vaccinated with rPRRS F (ORF3+5+7)

SOW No.	BEFORE WEANING				WEANING		POST-WEANING			
	No.	AGE DAYS	REF	Ab	No.	AGE DAYS	No.	AGE DAYS	REF	Ab
401426	10	11	443	≥640	10	32	10	38	443	-
			444	-					444	-
			445	≥640					445	160
			446	≥640					446	160
			447	≥640					447	80
			448	≥640					448	-
			449	≥640					449	160
			450	320					450	-
			451	160					451	80
			452	320					452	-

Sow No.: Reference of the sow

No.: Number of piglets; Ab: Antibodies; -: Negative

REF: Reference of the piglet

With the purpose of assessing the vaccines object of the trial, serological results as well as reproductive results have been evaluated. Table 10 shows some serological data, while Table 11 summarizes the reproductive data of the sows used in the trials, including information on the total number of piglets born, the number of piglets alive after the 1st week, the number of piglets weaned and the number of piglets of over 40 days of age.

Table 10

Summary of serological and reproductive data

VACCINE	SOW No.	SEROCONVERSION [IPMA] D 0	POST VAC.	POST INFECTION (7 days)
rPRRS C	400398	-	-	+
rPRRS C	400298	-	+	+
rPRRS D	400118	-	-	+
rPRRS D	400307	-	-	+
rPRRS E	314010	-	+	+
rPRRS E	313426	-	+	+
rPRRS E	400059	-	+	+
rPRRS F	313524	-	+	+
rPRRS F	401236	-	+	+
rPRRS F	401426	-	+	+
CONTROL	1	-	-	+
CONTROL	20	-	-	+

[-: Negative; + : Positive]

D O: Time of vaccination

Table 11

Summary of reproductive data

VACCINE	SOW No.	NO. OF PIGLETS			
		BORN	1st WEEK	WEANING	> 40 DAYS
CONTROL	1	17	0	0	0
	20	14	7	4	3
TOTAL		31	7	4	3
rPRRS C ORF3	400398	8	7	6	6
	400298	11	8	7	6
TOTAL		19	15	13	12
rPRRS D ORF5	400118	12	5	4	3
	400307	10	9	7	7
TOTAL		22	14	11	10
rPRRS E ORF 7	314010	12	3	2	1
	313426	6	3	3	3
	400059	12	1	0	0
TOTAL		30	7	5	4
rPRRS F ORF 3+5+7	313524	11	10	8	8
	401236	2	2	2	2
	401426	15	10	10	9
TOTAL		28	22	20	19

The results, in their totality, make it clear that in the case of vaccine rPRRS C, one sow serconverted (400298) and one did not (400398); in the case of vaccine D, none of the sows seroconverted; for vaccines E and F there is strong seroconversion due, chiefly, to the protein coded for ORF 7.

There is a favorable behavior in front of challenge, when the vaccinated animals are compared with those not vaccinated, enabling to assert positively that the recombinant vaccines object of the trial constitute an efficacious means for the prevention of PRRS.

It has been verified that vaccinated sows devoid of antibodies titrated with the IPMA technique are protected, which evidences that the said vaccines (rPRRS C and rPRRS D) are capable of inducing cellular immunity.

The efficacy of the vaccine was evaluated by comparing:

a) The percentage of piglets alive after the 1st week in contrast with the total number of piglets born,

b) the percentage of weaned piglets in contrast with the total number of piglets born, and

c) the percentage of piglets of over 40 days of age in contrast with the total number of piglets born.

Table 12 shows the data relative to the percentage of piglets alive after the 1st week, the percentage of piglets weaned, and the percentage of piglets of over 40 days of age in contrast with the total number of piglets born.

It has been verified that the animals devoid of antibodies, evaluated with the IPMA technique, are protected.

Table 12

Percentage of piglets alive after the 1st week, weaned, and of over 40 days in contrast with the total number of piglets born

VACCINE	% PIGLETS ALIVE 1st WEEK	% PIGLETS WEANED	% PIGLETS >40 DAYS
rPRRS C - ORF 3	79%	68.5%	63%
rPRRS D - ORF 5	63.6%	50%	45.5%
rPRRS E - ORF 7	23%	16.6%	13.3%
rPRRS F - ORF 3+5+7	78.6%	71.4%	67.8%
CONTROL	22.5%	12.9%	9.6%

DEPOSIT OF MICROORGANISMS

The recombinant baculoviruses obtained were deposited at the European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom in accordance with The Budapest Treaty of 1977.

The denomination and accession numbers of the recombinant baculoviruses are:

Denomination	ECACC Accession Number
AcNPV, PRRS2	V94021007
AcNPV, PRRS3	V94011325
AcNPV, PRRS4	V94021008
AcNPV, PRRS5	V94011326
AcNPV, PRRS6	V94011327
AcNPV, PRRS7	V94011328

All these baculoviruses were deposited on January 13,

1994, except for **AcNPV, PRRS2 (V94021007)** and **AcNPV, PRRS4 (V94021008)** which were deposited on February 10, 1994.

LEGEND FIGURES

Figure 3:

a) PRRSV genome
b) Size (Kb)
c) Clone number

Figure 9:

a) Antigen titration by ELISA
b) Absorbance at 405 nm
c) Antigen dilutions (1/)
d) Serum at 1/200

```
-- --- ---      Field
---+---+--       Experimental
---*---*--       Negative
```

Figure 10:

a) Serum titration by ELISA
b) Absorbance at 405 nm
c) Serum dilutions (1/)
d) Positive -- --- ---
   Negative --+--+--

Figure 11:

a) Field sera titration
b) Absorbance at 405 nm
c) Sow sera

PATENT CLAIMS

1. Recombinant proteins of the causative virus of porcine reproductive and respiratory syndrome (PRRS) characterized on account of the fact that they are chosen from any of the proteins coded by ORFs 2 to 7 of the virus PRRS-Olot.

2. Proteins as per patent claim 1, characterized on account of the fact that they comprise the amino acid sequences shown in Figure 2.

3. Proteins as per patent claim 1, characterized on account of the fact that they are obtainable by means of Genetic Engineering in a recombinant baculovirus expression system multiplied in a permissive host cell culture.

4. Proteins as per patent claim 3, characterized on account of the fact that such recombinant baculoviruses contain duly inserted and express, at least, the gen of a protein coded by ORFs 2 to 7 of the virus PRRS-Olot.

5. Proteins as per patent claim 3, characterized on account of the fact that such permissive host cell culture is a culture of permissive insect cells.

6. Proteins as per patent claim 3, characterized on account of the fact that they are obtainable by means of the expression of recombinant baculoviruses chosen from among:

Denomination	ECACC Accession Number
AcNPV, PRRS2	V94021007
AcNPV, PRRS3	V94011325
AcNPV, PRRS4	V94021008
AcNPV, PRRS5	V94011326
AcNPV, PRRS6	V94011327
AcNPV, PRRS7	V94011328

7. A procedure for the obtainment of recombinant PRRS-Olot proteins, coded by the genes contained in any of ORFs 2 to 7 of the said virus, which comprises the stages of:

a) preparation of the cDNA sequence, synthesized from the PRRS-Olot genomic RNA, to be inserted in a baculovirus; and

b) obtainment of recombinant baculoviruses that express the recombinant proteins corresponding to the inserted ORFs.

8. A procedure as per patent claim 7, characterized on account of the fact that the preparation of the cDNA sequence to be inserted comprises the stages of:

a.1 isolation and purification of the virus PRRS-Olot;

a.2 isolation of the PRRS-Olot viral RNA; and

a.3 synthesis of cDNA from the PRRS-Olot genomic RNA.

9. A procedure as per patent claim 8, characterized on account of the fact that the isolation of the virus PRRS-Olot is carried out by replication of the said virus on permissive cell cultures.

10. A procedure as per patent claim 8, characterized on account of the fact that the isolation of the PRRS-Olot viral RNA is carried out by adsorption onto an oligo $d(T)_{12}$-cellulose.

11. A procedure as per patent claim 8, characterized on account of the fact that the synthesis of cDNA, from the PRRS-Olot genomic RNA, is carried out by incubating the said RNA with the corresponding dNTPs, reverse transcriptase and either an oligo $d(T)_{12}$ or, alternatively, an oligonucleotide with formula 5´CGGGCTCGAGCCTTTGGCGA3´.

12. A procedure as per patent claim 8, characterized on account of the fact that the obtainment of recombinant baculoviruses expressing recombinant proteins that correspond to ORFs 2 to 7 of PRRS-Olot, comprise the stages of:

b.1 insertion of the corresponding ORF genes in baculovirus transfer vectors;

b.2 transfection of permissive host cells with the said transfer vectors that have inserted the corresponding ORF genes; and

b.3 selection of the recombinant baculoviruses that express the corresponding inserted ORF recombinant proteins.

13. A procedure as per patent claim 12, characterized on account of the fact that the said baculovirus transfer vector is vector pAcYM1.

14. A procedure as per patent claim 12, characterized on account of the fact that the transfection of the said permissive host cells for the replication of

recombinant baculoviruses is carried out with a mixture of DNA of the transfer vector that has inserted the corresponding ORF gene and DNA of the wild-type baculovirus.

15. A procedure as per patent claim 12, characterized on account of the fact that the said permissive host cells is an insect cell culture.

16. A procedure as per patent claim 12, characterized on account of the fact that the recombinant baculovirus obtained expresses a single recombinant protein of PRRS-Olot, chosen from any of the proteins coded by ORFs 2 to 7 of the said virus.

17. A procedure as per patent claim 12, characterized on account of the fact that the recombinant baculoviruses obtained are chosen from among:

Denomination	ECACC Accession Number
AcNPV, PRRS2	V94021007
AcNPV, PRRS3	V94011325
AcNPV, PRRS4	V94021008
AcNPV, PRRS5	V94011326
AcNPV, PRRS6	V94011327
AcNPV, PRRS7	V94011328

18. Recombinant baculoviruses, characterized on account of the fact that they express, at least, one recombinant protein corresponding to one of ORFs 2 to 7 of PRRS-Olot.

19. Recombinant baculoviruses as per patent claim 18, characterized on account of the fact that they

express a single recombinant protein of PRRS-Olot, chosen from among any of the proteins coded by ORFs 2 to 7 of the said virus.

20. Recombinant baculoviruses as per patent claim 18, characterized on account of the fact they are chosen from among:

Denomination	ECACC Accession Number
AcNPV, PRRS2	V94021007
AcNPV, PRRS3	V94011325
AcNPV, PRRS4	V94021008
AcNPV, PRRS5	V94011326
AcNPV, PRRS6	V94011327
AcNPV, PRRS7	V94011328

21. A vaccine suitable for the vaccination and protection of pigs in front of porcine reproductive and respiratory syndrome (PRRS), which comprises, at least, one recombinant protein corresponding to any of the proteins coded by ORFs 2 to 7 of PRRS-Olot and a suitable carrier or adjuvant.

22. A vaccine as per patent claim 21, characterized on account of the fact that the said recombinant proteins are obtainable by Genetic Engineering techniques in an expression system of recombinant baculoviruses multiplied in permissive host cell culture.

23. A vaccine as per patent claim 21, characterized on account of the fact that the recombinant baculoviruses that express the said recombinant proteins are chosen from among:

Denomination	ECACC Accession Number
AcNPV, PRRS2	V94021007
AcNPV, PRRS3	V94011325
AcNPV, PRRS4	V94021008
AcNPV, PRRS5	V94011326
AcNPV, PRRS6	V94011327
AcNPV, PRRS7	V94011328

24. Vaccine as per patent claim 21, characterized on account of the fact that said recombinant proteins are used partly purified.

25. Vaccine as per patent claim 21, characterized on account of the fact that the said antigenic phase contains a single recombinant PRRSV protein, selected from the group formed by the recombinant proteins coded by PRRS-Olot ORFs 3, 5 and 7.

26. Vaccine as per patent claim 21, characterized on account of the fact that the said antigenic phase is composed of insect cells infected with the same recombinant baculovirus expressing only one of the recombinant proteins coded by PRRS-Olot ORFs 2 to 7.

27. Vaccine as per patent claim 21, characterized on account of the fact that the said antigenic phase is composed of insect cells infected with different recombinant baculoviruses expressing, each one of them, only one of the different recombinant proteins coded by PRRS-Olot ORFs 2 to 7.

28. Vaccine as per patent claim 21 characterized on account of the fact that it contains an oily adjuvant.

29. Vaccine as per patent claim 28, characterized on account of the fact that the said oily adjuvant is composed of a mixture of Marcol RTM 52, Simulsol 5100 and Montanide RTM 888.

30. Vaccine as per patent claim 21, characterized on account of the fact that it contains an aqueous adjuvant.

31. Vaccine as per patent claim 21, characterized on account of the fact that it additionally contains cell response potentiator (CRP) substances such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, g-IFN, cell necrosis factor and similar substances.

32. A vaccine as per patent claim 21, characterized on account of the fact that the adjuvant is an adjuvant capable of modulating and immunostimulating cell response, such as MDP, ISCOM or liposomes.

33. A vaccine as per patent claim 21, characterized on account of the fact that it is capable of inducing cellular immunity in vaccinated animals.

34. A bi- or multivalent vaccine capable of preventing porcine reproductive and respiratory syndrome and another or other porcine infections, characterized on account of the fact that it comprises, at least, one recombinant protein corresponding to one of the proteins coded by any of the genes contained in any of ORFs 2 to 7 of PRRS-Olot, together with one or more porcine pathogens and a suitable carrier or adjuvant.

35. A vaccine as per patent claim 343, characterized on account of the fact that it includes, at least, one porcine pathogen selected from among the group formed by Actinobacillus pleuropneumoniae, Haemophilus parasuis, Porcine parvovirus, Leptospira, Escherichia coli, Erysipelothrix rhusiopathiae, Pasteurella multocida, Bordetella bronchiseptica, Porcine respiratory coronavirus, Rotavirus or in front of the pathogens causative of Aujeszky's Disease, Swine Influenza or Transmissible Gastroenteritis.

36. A passive vaccine suitable for the vaccination and protection of pigs in front of porcine reproductive and respiratory syndrome (PRRS) characterized on account of the fact that it contains antibodies obtained by means of the immunization of animals with, at least, one recombinant protein corresponding to one of the proteins coded by any of the genes contained in any of ORFs 2 to 7 of PRRS-Olot and a suitable carrier or adjuvant.

37. A diagnostic kit for the detection of the presence of antibodies that specifically identify PRRSV in a biological sample, such as blood, serum, sputum, saliva or milk from pigs, comprising, at least, one recombinant protein corresponding to one of ORFs 2 to 7 of PRRS-Olot and suitable detection means.

38. A diagnostic kit as per patent claim 37, characterized on account of the fact that the said recombinant proteins are obtainable by Genetic Engineering in an expression system of recombinant baculoviruses multiplied in permissive host cell culture.

39. A diagnostic kit as per patent claim 38, characterized on account of the fact that the recombinant baculoviruses expressing the said recombinant proteins are selected from:

Denomination	ECACC Accession Number
AcNPV, PRRS2	V94021007
AcNPV, PRRS3	V94011325
AcNPV, PRRS4	V94021008
AcNPV, PRRS5	V94011326
AcNPV, PRRS6	V94011327
AcNPV, PRRS7	V94011328

40. A diagnostic kit for the detection of the presence of PRRSV in a biological sample, such as blood, serum, sputum, saliva, tissue or milk, from pigs, comprising antibodies that specifically identify the PRRSV obtained by immunizing animals with, at least, one recombinant protein corresponding to one of ORFs 2 to 7 of PRRS-Olot and suitable detection means.

The Patent Office

Application No:	GB 9509392.8	**Examiner:**	Dr. N.R. Curtis
Claims searched:	1-40	**Date of search:**	26 July 1995

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.N): C3H (HB7P; HB7V, HB7T)

Int Cl (Ed.6): C07K 14/08

Other: ONLINE: WPI; BIOTECH (DIALOG); CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
Y	EP 0,595,436 A2 (Solvay Animal Health) (See page 9, lines 22-39; page 10, lines 25-33, 40-49)	1-5, 7-16, 18, 19, 21, 22, 24, 26-38, 40
Y	WO 92/21375 A1 (Stichting Centraal Diergeneeskundig Institut) (See page 3, lines 13-31; page 4, lines 13-35, page 7, lines 29-33)	1-5, 7-16, 18, 19, 21, 22, 24, 26-38, 40
Y	ANIMAL PHARM, No. 293, 28 Jan. 1994, "Cyanimid Spain studying 2nd-generarion PRRS vaccine", page 23	1-5, 7-16, 18, 19, 21, 22, 24, 26-38, 40
Y	VETERINARY MICROBIOLOGY, Vol. 33, 1992, Plana et al. "Porcine epidemic abortion and respiratory syndrome (mystery swine disease). Isolation in Spain of the causative agent and experimental reproduction of the disease", pages 203-211).	1-5, 7-16, 18, 19, 21, 22, 24, 26-38, 40

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

68 The Patent Office

Application No: GB 9509392.8 **Examiner:** Dr. N.R. Curtis
Claims searched: 1-40 **Date of search:** 26 July 1995

Category	Identity of document and relevant passage	Relevant to claims
Y	ANIMAL PHARM, No. 238, 1991, "Cyanimid reports on isolation of PRRS virus", page 20	1-5, 7-16, 18, 19, 21, 22, 24, 26-38, 40

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.